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Characteristics of an Endonuclease Associated with Adenovirus Types 2 and 12

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Characteristics of an Endonuclease Associated with Adenovirus Types 2 and 12

A thesis submitted to the Faculty of The Rockefeller University
in partial fulfillment of the requirements
for the degree of Doctor of Philosophy

by

Byron T. Burlingham

Approval for publication
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15 May, 1970
The Rockefeller University
New York, New York

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PREFACE

The majority of the viruses of vertebrates appear to have little or no oncogenic potential. However, the normal manifestation of infection by a relatively few viruses is a benign or malignant tumor. Certain of the adenoviruses infecting hamsters produce malignant tumors. These tumors do not produce the adenovirions which caused the tumor. But these malignant cells do contain virus gene products many generations after the virus first interacted with the cell. Thus the viral genome or a portion of it must be present in the cell. The experiments described here are a small addition to the work of many who have sought to understand the fate and the metabolism of the viral DNA in transformed cells.

I wish to express my gratitude to Dr. Detlev W. Bronk, whose foresight and energies created the graduate program at The Rockefeller University, and whose kindness provided the opportunity for me to participate in this program.

I am also grateful to President Frederick Seitz, and Dean Frank Brink, Jr. for their contributions to the continuation of this unique and stimulating educational opportunity. My research adviser, Dr. Walter Doerfler, has provided expert guidance and personal encouragement while I pursued this research. His continuing patience and understanding helped me to pursue a self-determined course. His ready advice, assistance, critical judgment and experience has been valuable at all stages of this project. It has been a rewarding personal and professional experience to work in his laboratory.

I am grateful to Dr. Igor Tamm who served as my faculty adviser during my first years. I am also thankful to all the members of his department for their friendship and intellectual stimulation. I am particularly indebted to Mrs. Faith Arthur and Mrs. Agnes Morra for preparation of special glassware; to Miss Huguette Viguet for preparation of voluminous amounts of media; to Mr. Arthur Cruger for his aid in performance of

animal studies; to Miss Ruth Mandelbaum and the Illustrations Service for their artistry in preparing the illustrations; to Miss Mary Arakelian who typed a portion of this manuscript; and especially to Miss Elizabeth Langmore who patiently deciphered my cryptic handwriting, typed much of this manuscript, and faithfully prepared the final copy of this thesis.

I am especially grateful for the assistance of Miss Karen Leider who donated her summer vacations and many college holidays to assist with the experiments. My devoted wife, Susan, has encouraged me, helped perform many experiments, and proofread this manuscript. She has patiently endured many fallen souffles, curdled Stroganoff's and sleepless nights during the last four years.

ABBREVIATIONS AND GLOSSARY

Å	- Ångstrom units, 0.1 nm.
A_{260}	- Absorbance at 260 nm in optical density units.
A_{280}	- Absorbance at 280 nm in optical density units.
Ad 2	- Adenovirus type 2.
Ad 12	- Adenovirus type 12.
BHK21	- Baby hamster kidney cells, a continuous cell line isolated by Stoker and Macpherson (1964) from kidneys of newborn hamsters.
dAT	- Alternating copolymer of deoxyadenylic acid and deoxythymidylic acid.
dG:dC	- Polymer composed of one strand polydeoxyguanylic acid and one strand polydeoxycytidylic acid.
DNA	- Deoxyribonucleic acid.
DNAse	- Deoxyribonuclease.
HEK	- Human embryonic kidney cells. A primary cell line derived from kidneys of human embryos.
HEL	- Human embryonic lung cells. A primary cell line derived from lungs of human embryos.
HeLa	- A continuous cell line isolated by Gey (Scherer et al., 1953) from an epidermoid carcinoma of the cervix.
in vitro	- Refers to experiments or events occurring in a defined system within a test tube.
in vivo	- Refers to experiments or events occurring in cells, usually in culture.
KB	- A continuous cell line isolated by Eagle (1955) from an epidermoid carcinoma of the mouth.
M	- Molecular weight in Daltons.
mRNA	- Messenger RNA.

OD - Optical density.

ODU - Optical density units.

PBS - Phosphate buffered saline (Dulbecco and Vogt, 1954).

PFU - Plaque forming units.

p.i. - After infection.

RNA - Ribonucleic acid.

$\underline{S}$ - Sedimentation coefficient in Svedbergs (10^{-13} sec).

$\underline{S}^{\circ}_{20,w}$ - Sedimentation coefficient corrected to sedimentation in water at 20°C.

SDS - Sodium dodecylsulfate.

SEM - Standard error of the mean.

TCA - Trichloroacetic acid.

Tris - Trishydroxyaminomethane (2-amino-2(hydroxymethyl)-propane-1,3-diol).

tRNA - Transfer RNA.

$\bar{V}$ - Partial specific volume.

$\bar{x}$ - Mean of x.

η - Viscosity in centipoises, or refractive index.

ρ - Buoyant density - g/cm^3.

ω - Angular velocity - radians/sec.

ABSTRACT

Adenovirus type 2 productively infects human cells and hamster cells. Adenovirus type 12 productively infects human cells but abortively infects hamster cells. The metabolism of the DNA of these adenoviruses was determined in both human cells and hamster cells. The adenovirus DNA, both the parental DNA and the newly synthesized DNA, is found as three species within the cells. The species which sedimented rapidly is mainly cellular DNA which contains some integrated viral DNA. The species which cosediments with DNA extracted from the mature virions is homogeneously distributed. This species represents the intact viral genome. The species of DNA which sediments slowly is homogeneously distributed, hybridizes only to viral DNA and represents segments of the adenovirus genome about one-fourth the size of the intact genome. The amount of each of these three species of DNA varies during the growth cycle of the adenovirus. There is a pattern of metabolism; the intact viral DNA is cleaved to the slow sedimenting DNA, the slow sedimenting DNA appears as a precursor which is integrated into the cellular DNA. Data from experiments with chemical inhibitors of macromolecular syntheses suggest that the slow sedimenting DNA is formed by a presynthesized endonuclease.

Extracts of cells infected with adenovirus contain the endonuclease activity. The endonuclease appears in the cells as both an early enzyme and a late enzyme. The endonuclease which appears early after infection correlates directly with the multiplicity of infection but is not effected by inactivation of the viral genome. The endonuclease which appears late after infection is not effected by the multiplicity of infection if more than one infectious particle per cell is used. The endonuclease which appears late is not found in cells infected by virus which contain inactivated genomes. These results suggest that the endonuclease which appears early is carried into the cell as a portion of the virion

and the endonuclease which appears late is newly synthesized as a virus-coded protein. The synthesis of endonuclease corresponds with the synthesis of structural proteins.

Purified adenovirions have endonuclease activity when they are incubated with DNA in vitro. The endonuclease is inactivated by group-specific antiserum. This also suggests that the endonuclease is a virus-coded protein. The endonuclease made only double-strand scissions at specific sites in the adenovirus DNA.

Capsid subunits purified from KB cells infected with adenovirus type 2 were assayed for endonuclease activity. Only the penton, composed of a penton base and a fiber, cleaves the DNA substrate. The fiber does not compete with the penton for the substrate. The endonuclease was specifically inactivated by treating the penton with trypsin and group-specific antiserum. These results indicate that only the five, presumably identical, polypeptides of the penton base are the endonuclease. Thus the penton base is a structural subunit of the adenovirion and an endonuclease.

The penton endonuclease cleaves native DNA about 20 times faster than denatured DNA. Glucosylated DNA, double-stranded RNA, and single-stranded RNA are not cleaved. The site of attack on the DNA molecule apparently is a guanine-cytosine rich region. The endonuclease is inhibited by polydeoxyguanylic acid - polydeoxycytidylic acid but not by alternating copolymer of deoxyadenylic acid - deoxythymidylic acid.

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I. General Introduction to Adenoviruses

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I. GENERAL INTRODUCTION TO ADENOVIRUSES

A. Discovery and Isolation

1. Human adenoviruses. Adenoviruses were first isolated by Rowe et al. (1953) from human adenoidal tissues which had been removed surgically. After prolonged culture, some of the epithelial cells degenerated spontaneously. Thirteen isolates of an agent from the medium of these cultures were passaged and caused similar degeneration in normal cell cultures of human embryonic epithelium and HeLa cells.

Adenovirus isolates are classified into serotypes on the basis of specific neutralization of virus infectivity by antiserum. Adenovirus types 1, 2, 5 and 6 were isolated from human adenoids and typed in this way. Subsequently, Huebner et al. (1954) demonstrated that serologically related viruses which produced characteristic intranuclear inclusion bodies are present in the tonsils, in nasopharyngeal and conjunctival secretions and in anal excretions of persons undergoing natural infection with these agents.

Nearly simultaneously, Hilleman and Werner (1954) isolated a cytopathic agent, which later was identified as adenovirus type 4, from army recruits with primary atypical pneumonia. There are now 32 serotypes of human adenoviruses recognized; however, most do not appear to be pathogenic for humans.

2. Adenoviruses from other animals. Adenoviruses were also isolated from the respiratory tracts of monkeys, dogs, cattle, swine, mice and birds. Seven subgroups of adenoviruses were established by Pereira et al. (1963), one subgroup was designated for each species from which adenovirus was isolated. The adenoviruses from these animals were usually isolated from adenoidal or renal tissue, feces or respiratory tract secretions. The adenoviruses from these species have a narrow host range and often will only replicate in tissue from very closely related species.

B. Characteristics of Adenoviruses

The original criteria which characterized the adenoviruses were established by Rowe et al. (1955). These criteria were: ether resistance,

cytopathic effect on epithelial cells in tissue culture, production of a group specific soluble antigen, and lack of pathogenicity for laboratory animals.

These criteria have been extended to a more complete, modern definition of the adenovirus group by The Virus Subcommittee of the International Nomenclature Committee (Pereira et al., 1963). The proposed criteria fall into seven categories:

1. Chemical and physical properties. The adenovirions contain DNA as the only nucleic acid. The virions are icosahedra with a uniform structure and have a diameter of 60-85 nm. The adenoviruses are very stable at low temperatures but are readily inactivated at 56°C. The adenoviruses are stable between pH 4 and 10. A more detailed description of the chemical and physical properties will be discussed under structure and composition of adenoviruses (Section I. C.).

2. Growth. The adenoviruses are readily propagated in primary cultures of epithelial cells derived from naturally susceptible animals. Replication and maturation of adenoviruses are completed within the nucleus of infected cells (Brandon and McLean, 1962). Human adenoviruses are readily propagated in primary cultures or cell lines derived from human tissues. Epithelial cells are more susceptible than fibroblasts. In tissue culture, most human adenoviruses cause cytopathic effects in cells from sources other than human, but serial propagation of the virus can be demonstrated in only a limited number of cell lines. Human adenoviruses cannot be grown in embryonated eggs. A detailed description of the adenovirus growth cycle will be discussed in Section I. D., and oncogenesis and transformation will be discussed in Section I. E.

3. Antigenic characteristics. Most serotypes are clearly distinguished by virus neutralization tests although cross-reactions are observed between some types. All adenoviruses contain a group specific antigen, separate from the virus particles, called the soluble antigen. The soluble antigen is detectable by complement fixation, gel-diffusion precipitation and indirect hemagglutination. Some adenoviruses produce at least three soluble

antigens differing from each other in physicochemical properties and in serological specificity.

4. Hemagglutination. Most adenoviruses agglutinate erythrocytes of some animals. Hemagglutination is inhibited by type specific antiserum. Human adenoviruses have been subdivided by Rosen (1960) into four groups according to their ability to agglutinate rat and rhesus monkey erythrocytes. These subgroups are listed in Table 1. Some types of adenovirus are also able to agglutinate human, grivet monkey, guinea pig and mouse erythrocytes. The subgroups of adenoviruses based on hemagglutination also have other common properties, such as antigenic relationship and similar fiber structure.

5. Habitat. Adenoviruses are found in the upper respiratory, digestive and occular systems of susceptible animals. Adenovirus may persist for prolonged periods in lymphatic organs and kidneys, usually with no known clinical manifestations.

6. Pathogenicity. Adenoviruses are frequently associated with acute upper respiratory illness. The host range is usually limited to naturally susceptible animals although some types of adenovirus may cause experimental infections, usually without symptoms, in laboratory or domestic animals.

Several types of human adenoviruses cause respiratory illness and gastroenteritis. Acute respiratory disease is caused mainly by Ad 4 and 7 and is almost exclusively seen in military recruits. Pharyngitis and pharyngoconjunctival fever is principally caused by Ad 3, but Ad 1, 2, 4, 5, 7, 9 and 21 also cause this illness. Conjunctivitis is caused by Ad 3 and keratoconjunctivitis is caused by Ad 8 and has serious sequelae with 1 to 10% of the patients with persistent impairment of vision. A prominent clinical feature of all these illnesses is lymphadenopathy, hyperplasia of tonsillar and adenoidal tissue and lymphocytosis.

Huebner et al. (1954) have postulated that adenoviruses can cause a low-grade chronic infection of adenoidal tissue, leading to hypertrophy. This is supported by frequent isolations of various types of adenoviruses from apparently normal tonsillar and adenoidal tissue which has been

Table 1

Subgroups of Human Adenoviruses
Based on Hemagglutination Properties[a]

Group	Serotypes	Hemagglutination of erythrocytes	
		Rhesus monkey	Rat
1	3, 7, 11, 14, 16 20, 21, 25, 28	+	0
2	8-10, 13, 15, 17, 19, 22-24, 26, 27, 29[c], 30[c]	0 (+)[b]	+
3	1, 2, 4-6	0	Partially
4	12, 18, 31[c]	0	0

[a] Taken from Rosen (1960).

[b] Types 9, 13, and 15 also agglutinate rhesus monkey cells but at a lower titer than rat cells.

[c] Added from Norrby (1968).

removed surgically. Only the canine adenovirus, the etiologic agent of infectious canine hepatitis, has been clearly shown to be responsible for a naturally occurring disease in lower animals.

7. Natural transmission. Transmission is usually from the upper respiratory tract or conjunctiva. Some types of adenoviruses are also transmitted by means of contaminated feces and urine. There is no evidence of transmission by vectors. Transmission is horizontal only, and there is no evidence for vertical transmission.

C. Structure and Composition of Adenoviruses

1. Morphological and physical characteristics of the virion. Morgan et al. (1956) reported that the Ad 4 virions seen in thin sections of infected cells or in preparations of purified particles had a diameter of 60-90 nm. Hilleman et al. (1955) determined that the diameter of Ad 4 was 109 ± 6 nm by velocity sedimentation and 80-120 nm by membrane filtration. Green (1962) reported a sedimentation coefficient of 795 S for Ad 2 and calculated a corresponding molecular weight of 87 million Daltons. Chemical determinations by Green and Piña (1964) yielded a molecular weight of 145 million Daltons for Ad 2.

Negatively stained preparations of Ad 5 by Horne et al. (1959), and by Wilcox et al. (1963) and of Ad 7 by Dales (1962) showed icosahedral virus particles consisting of 252 subunits, called capsomeres, arranged in 5:3:2 cubic symmetry. The capsomeres appeared as polygons with a 70-80 Å diameter. The typical adenovirus morphology was also found in the simian adenoviruses by Archetti and Steve-Bocciarelli (1963), infectious canine hepatitis virus by Davies et al. (1961) and the avian adenovirus Gal by Macpherson et al. (1961).

The adenovirion, on thin sections, contains a dense central core, the nucleoid, and an outer protein layer, the capsid (Morgan et al., 1956). The morphology of Ad 2 has been described by Pettersson et al. (1967). The capsid was negatively stained with phosphotungstic acid. The capsid has a sixfold symmetry and is composed of two types of capsomeres: 1) 240 hexons, the subunits on the facets of the icosahedron,

and 2) 12 pentons. Hexons are surrounded by six identical subunits. Valentine and Pereira (1965) and Norrby (1966) demonstrated that the pentons were found at the vertices of the icosahedron and were surrounded by five identical hexon subunits.

The penton is made of two parts, the penton base and the fiber. The association of the fiber with the penton was first suggested for Ad 5 by Wilcox et al. (1963) and was clearly demonstrated by Valentine and Pereira (1965) for Ad 5, by Norrby (1966) for Ad 3, by Pettersson et al. (1967) for Ad 2 and by Gelderblom et al. (1967) for Ad 13, 15, and 19. These authors found that fibers were 100-300 Å long, depending on the serotype, and 15 Å wide. The peripheral knob had a diameter of 40 Å. The morphology of the adenovirus capsid and the capsid subunits and their relationship to each other are illustrated in Figure 1.

2. Morphological and physical characteristics of the capsid subunits. The adenovirus nucleoid or core appears amorphous on thin sections and the only morphological subunits which can be resolved are the hexons, penton bases and fibers found in the capsid. These subunits have been isolated and partially purified by Pereira et al. (1959) using immunoelectrophoresis and by Klemperer and Pereira (1959), Philipson (1960), and Wilcox and Ginsberg (1961) using DEAE chromatography. These partially purified adenovirus subunits have been characterized and studied.

There are 240 hexons covering the facets of each adenovirus particle. The hexon has been identified as the former "A" antigen or group antigen (see Fig. 1) by Klemperer and Pereira (1959) and Allison et al. (1960). Pettersson et al. (1967) examined purified Ad 2 hexons contrasted with uranyl acetate in the electron microscope. The hexons were 80-110 Å in diameter with a mean diameter of 95 Å, and contained a central hole of 25 Å diameter. In many electron micrographs the contour of the hexons appeared hexagonal. The detailed structure of the hexon was complex and it could not be described as a hollow cylinder as suggested by Dales (1962) or as a sphere as suggested by Valentine and Pereira (1965).

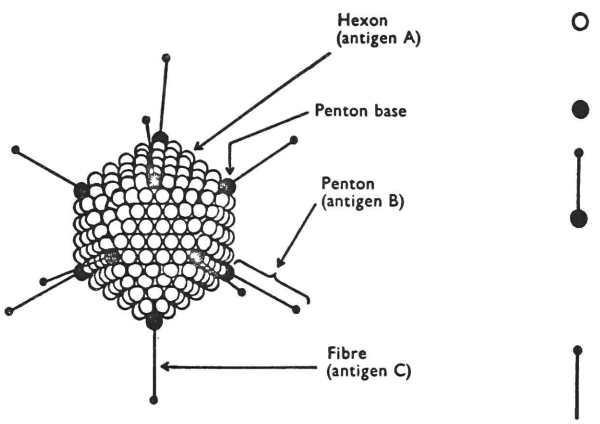

Figure 1: Morphology of the adenovirus capsid and the capsid subunits. The icosahedral virion has a diameter of about 720 Å. Each virion contains 240 hexons and 12 pentons. Each penton is composed of a penton base and a fiber. The length of the fibers varies from 100 to 300 Å depending on the type of adenovirus. Figure 1 was adapted from Valentine and Pereira (1965).

Pettersson et al. (1967) found that the Ad 2 hexon had a sedimentation coefficient of 12.2 ± 0.2 S and a diffusion coefficient of 2.6×10^{-7} cm^2/sec. The partial specific volume calculated from the amino acid composition was 0.720 cm^3/g. When these values were used, the molecular weight of the hexon was estimated as 400,000 Daltons.

Valentine and Pereira (1965) counted the hexons and pentons from dissociated, isolated Ad 5 virions and found 22 hexons per penton. Morphological evidence indicated that each virion contains 12 pentons, one at each vertex of the icosahedron. Previous studies by Wilcox and Ginsberg (1963b) indicated that the hexon antigen was 16 to 32 times more plentiful than the penton antigen. Russell et al. (1967b) have demonstrated that heating Ad 5 virions to 56°C for 1 min will release the penton bases and the adjacent 5 hexons from the capsid and leave the remaining 180 hexons in an icosahedron with holes at the vertices.

The Ad 2 penton, consisting of a penton base and a fiber, has been isolated and purified by Pettersson and Höglund (1969). The former "B" antigen or early cytopathic component has been identified as the penton base and the former "C" antigen or type specific antigen has been identified as the fiber by Klemperer and Pereira (1959), Philipson (1960), and Wilcox and Ginsberg (1961). The free penton bases have not been isolated from infected KB cells; however, Norrby and Skaaret (1967) and Neurath et al. (1968) have demonstrated the release of free penton bases after treatment of pentons from Ad 3, 4, and 7 with guanidine-HCl or formamide.

Pettersson and Höglund (1969) contrasted Ad 2 pentons isolated from KB cells with uranyl acetate. A structure composed of a globular head and a threadlike tail was resolved. The head or penton base was 90 Å in diameter and showed a central cavity. The outline of the penton base was irregular but a pentagonal contour was often observed. The fiber was 200 Å long. The sedimentation coefficient of the penton was 10.5 ± 0.05 S. The molecular weight was estimated to be 370,000 Daltons using the Archibald method and 400,000 Daltons by the Yphantis method.

The Ad 2 fiber was isolated by Pettersson et al. (1968). The fiber was contrasted with phosphotungstic acid and examined in the electron microscope. The mean length of the fiber was 200 Å, the width was 15-20 Å and the terminal knob was 40 Å in diameter. The fiber had a sedimentation coefficient of 6.1 S. This corresponds to a molecular weight of 70,000 Daltons. The length of the fiber varies with the type of adenovirus. Valentine and Pereira (1965) reported Ad 5 fibers to be 240 Å long, Norrby and Wadell (1967) reported Ad 4 fibers to be 170-180 Å long, Norrby (1968) found Ad 9 fibers to be 120-150 Å long and Gelderblom et al. (1967) reported fibers for Ad 13, 15, and 19 to be 160-190 Å long.

3. Proteins of the adenovirion. The adenovirion is composed of only DNA and protein. Green et al. (1967b) found 1.52×10^8 Daltons of protein per Ad 2 virion. All analyses for lipid, carbohydrate and RNA have been negative. These determinations were sensitive enough to detect these components if they had represented more than 0.01% of the mass of the virion. In addition, treatment of the virion with ether by Huebner et al. (1954), chloroform by Feldman and Wang (1961), fluorocarbons by Wilcox and Ginsberg (1963a), or deoxycholate by Ginsberg (1964) had no effect on infectivity.

Polasa and Green (1967) have determined the amino acid composition of five purified adenoviruses and also of KB cells. The amino acid composition of Ad 2, Ad 12, and KB cells are listed in Table 2. The recovery of amino acids from the adenovirus hydrolysates based on total nitrogen determinations was close to 100%. Thus, there are no appreciable amounts of unusual amino acids present. The large amount of ammonia suggested that nearly all of the glutamic acid and aspartic acid is present as glutamyl and asparaginyl residues. The amino acid composition of different adenovirus serotypes was very similar; however, there were large differences between the amino acid composition of KB cell and of adenovirus protein. Grossly, the amino acid composition of adenovirus proteins is similar to those of RNA containing animal viruses like poliovirus, as reported by Levintow and Darnell (1960), except the adenoviruses contain twice as much arginine as poliovirus.

Table 2

Amino Acid Composition of Adenovirus Types 2 and 12 and of KB Cells[a]

Amino acid	Ad 2	Ad 12	KB cells
Aspartic acid	11.8	11.5	9.4
Threonine[b]	6.9	7.2	5.5
Serine[b]	6.7	7.0	5.9
Glutamic acid	9.0	8.8	12.0
Proline	7.2	7.4	5.2
Glycine	7.8	8.4	7.4
Alanine	9.0	9.6	8.1
Valine	6.1	5.8	7.2
Half-cystine	0.3	0.5	1.1
Methionine[b]	2.3	2.6	2.4
Isoleucine	3.4	3.3	5.2
Leucine	7.4	7.1	8.9
Tyrosine[b]	4.4	4.1	3.0
Phenylalanine	3.8	3.7	3.9
Ammonia	21.4	22.0	9.8
Lysine	4.4	4.1	7.6
Histidine	1.6	1.6	2.1
Arginine	7.9	7.3	5.2
Tryptophan[c]	1.2	1.4	0.5

[a] Polasa and Green (1967). These values represent the number of residues per 100.

[b] Extrapolated to zero time of hydrolysis.

[c] Determined by the method of Spies and Chambers (1949).

Maizel et al. (1968a,b) have analyzed the proteins of Ad 2, 7, and 12 virions, soluble antigens (capsid subunits), cores and incomplete particles. By polyacrylamide gel electrophoresis of dissociated Ad 2, 7, and 12 virions, nine different polypeptides can be resolved. The polypeptide patterns of the virion were compared with polypeptide patterns obtained from subunits of the virion. Thus most of the nine polypeptide peaks of the virion could be correlated with substructures of the virion. The data in Table 3 identify and characterize the subunits and polypeptides of the adenoviruses. The polypeptides from a single subunit are presumed to be identical. The polypeptide patterns of these three adenoviruses revealed a generally similar peptide pattern. However, there were distinct differences in size and amino acid ratios between the corresponding peptides of these three types of adenovirus.

Pettersson et al. (1967, 1968, 1969) have determined the amino acid composition of the capsid subunits from Ad 2. The data of this analysis and the amino acid composition of the Ad 2 virion are listed in Table 4. Although the virion is relatively rich in basic amino acids, the capsid proteins are remarkably poor in arginine. It is also striking that the capsid proteins are relatively rich in hydroxyl amino acids. Also, the capsid proteins contain a rather constant proportion of each amino acid.

Laver et al. (1967) labeled the N-terminal amino acids of the proteins from eight adenoviruses with phenylisothiocyanate-^{35}S and found that only alanine and glycine contained the label. For most adenoviruses the ratio of alanine to glycine was 2.5 but for Ad 2 the ratio was 3.6 and for Ad 5 it was 5.5. The proteins with N-terminal alanine were associated only with the DNA. However, some proteins associated with the DNA also contained N-terminal glycine.

The nucleoprotein core has been partially characterized by Prage et al. (1968). The pentons and some of the hexons were removed from the virion by dialysis against 0.01 M glycine, 0.001 M tris-HCl pH 7.2 and 0.01% Triton X-100. The resulting particles had a buoyant density of 1.365 g/cm^3 in CsCl and were 100-fold less infectious than intact particles. Sixteen cycles of freezing and thawing quantitatively removed the hexons.

Table 3

Characteristics of Morphological Subunits of Adenovirions[a]

Morphological subunits			Polypeptides	
Name	Number per virion	Molecular weight	Number per subunit	Molecular weight
Hexon	240	360,000[a]	3[g]	120,000[g,h]
		400,000[b]		
Penton base	12	370,000[c]	5	70,000
Fiber	12	70,000[d,e]	1	62,000
Core				
DNA		24,400,000[f]		
Proteins		29,000,000		
V			180	44,000
VI and VII			890	24,000
VIII and IX			400	13,000
X			800	7,500

[a] Maizel et al. (1968b).

[b] Pettersson et al. (1967).

[c] Pettersson and Höglund (1969).

[d] Valentine and Pereira (1965).

[e] Pettersson et al. (1968).

[f] Green et al. (1967b).

[g] Recent data from Philipson (personal communication) indicates that each hexon consists of 6 peptides of 60,000 Daltons per hexon. The values of Maizel et al. (1968a,b) represent dimers due to incomplete dissociation.

[h] Recent data from Ginsberg (personal communication) disputes these molecular weight values. With Ad 5 hexons, the molecular weight of the hexon polypeptide seems to be 25,000 Daltons (Velicer and Ginsberg, 1970).

Table 4

Amino Acid Composition of Adenovirus Type 2 Virions, Their Capsid Subunits and Core Proteins[a]

Amino acid	Virion[b]	Fiber[c]	Penton[d]	Hexon[e]	AS_1[f]	AS_2[f]	AS_3[f]
Lysine	4.4	6.0	5.1	4.4	4.4	5.9	8.1
Histidine	1.6	0.8	1.6	1.7	3.2	2.2	2.0
Arginine	7.9	1.6	3.9	4.7	11.7	7.5	4.5
Aspartic acid	11.8	12.8	12.9	14.4	7.8	10.0	12.0
Threonine[g]	6.9	11.0	9.1	7.3	7.2	5.9	5.2
Serine[g]	6.7	12.1	9.9	7.2	13.7	6.1	6.4
Glutamic acid	9.0	7.2	8.5	9.7	6.5	10.0	10.9
Proline	7.2	5.2	6.4	6.5	4.7	6.0	5.0
Glycine	7.8	9.5	7.7	7.8	11.7	8.2	10.2
Alanine	9.0	7.1	7.6	7.5	12.4	8.8	9.3
Valine	6.1	5.4	6.0	5.4	5.8	6.1	6.3
Methionine[g]	2.3	1.2	1.5	2.3	0.3	1.9	0.0
Isoleucine	3.4	5.2	4.2	3.4	2.1	4.6	4.8
Leucine	7.4	9.6	8.4	7.5	3.6	7.9	8.1
Tyrosine[g]	4.4	2.1	2.4	5.0	1.6	3.1	2.0
Phenylalanine	3.8	2.2	3.7	4.3	3.2	3.5	3.7
Tryptophan[h]	1.2	1.2	1.1	0.9	ND	1.2	1.5
Half-cystine	0.2	ND[i]	ND	ND	ND	1.1	ND

[a] Given as number of residues per 100.

[b] Polasa and Green (1967).

[c] Pettersson et al. (1968).

[d] Pettersson et al. (1969).

[e] Pettersson et al. (1967).

[f] Boulanger et al. (1970). AS_1, AS_2 and AS_3 designate three acid soluble "core proteins" (see text).

[g] Extrapolated to zero time of hydrolysis.

[h] Determined spectrophotometrically according to Bencze and Schmidt (1957).

[i] ND = none detected.

The resulting particles had a buoyant density of 1.38 g/cm^3 and were free of capsid proteins. These particles were considered to be "cores"; however, they were not characterized or examined by electron microscopy. These cores are considerably less dense than the cores described by Lonberg-Holm and Philipson (1969).

The acid-soluble proteins extracted by Prage et al. (1968) from such "cores" comprised 15-20% of the total viral protein. These acid soluble proteins contain about 20% arginine, and therefore were classified as histones. Two proteins extracted from the "cores" were isolated and characterized. The molecular weight of these two proteins is about 20-30,000 Daltons, and they formed about 30-40% of the core proteins.

Boulanger et al. (1970) have also analyzed the core proteins. Three major groups of proteins were extracted with 0.25 N HCl from the cores of Ad 2. These proteins were resolved by polyacrylamide gel electrophoresis and designated AS_1, AS_2 and AS_3. AS_1 contains one major protein with glycine as the N-terminal residue. AS_2 contains three major proteins and the N-terminal amino acids were glycine, alanine and threonine. These three proteins of AS_2 probably correspond to proteins V, VI, and VII described by Maizel et al. (1968). AS_3 had several slow migrating components which have not been characterized since AS_3 was contaminated with a small amount of hexon proteins. These core proteins of Ad 2 seem relatively poor in N-terminal alanine to correlate well with the results described by Laver et al. (1967).

4. Adenovirus DNA. The DNA content of the adenovirion varies, depending on the serotype, from 12.9 to 14 percent of the virus particle (Green, 1962; Green and Piña, 1963a; Ginsberg, 1964). The DNA of adenoviruses have been physically characterized by Green (1962), Green and Piña (1963a), and Green et al. (1967b). The buoyant density of the DNA was determined by equilibrium sedimentation. The size was determined by velocity sedimentation in neutral and alkaline solutions and by electron microscopy. All of the DNA species from adenovirions were single-stranded linear molecules

with molecular weight from 20-25 million Daltons (see Table 5). Furthermore, reannealing of denatured DNA or digestion with exonuclease III followed by annealing according to the method of MacHattie et al. (1964) rarely gave circular DNA, thus there is probably no terminal redundancy in these genomes (Green et al., 1967b).

Green and Piña (1963b, 1964) have correlated the properties of the adenovirus DNA with the ability of the adenovirion to induce tumors in newborn hamsters (see Table 6). These investigators found that the adenoviruses can be divided into three groups. Group A is highly oncogenic for hamsters and the DNA of the adenoviruses in this group contains 47-49% guanine plus cytosine. Group B is weakly oncogenic and the DNA of the adenoviruses in this group contains 50-55% guanine plus cytosine. Group C is nononcogenic for hamsters and the DNA of the adenoviruses in this group contains 57-59% guanine plus cytosine.

Doerfler and Kleinschmidt (1970) have derived a physical map of the Ad 2 genome from the thermal melting patterns as determined by electron microscopy with the technique described by Inman (1966). Figure 2 illustrates the melting patterns observed. The guanine-cytosine rich regions melt at a higher temperature than the adenine-thymine rich regions. From the melting map, it is apparent that there are three regions which are relatively richer in guanine-cytosine residues. These regions are located at 0.25, 0.50, and 0.70 the length of the Ad 2 DNA molecule. These regions divide the Ad 2 genome into nearly equal fragments, each approximately one-fourth the total length of the intact DNA. The unique melting patterns and the guanine-cytosine rich regions are more apparent in the frequency distribution of the melted regions represented in Figure 3.

Smith (1964) has reported a circular form of Ad 2 DNA isolated from purified particles. However, these circular forms of Ad 2 DNA had a mean length of only 2.3 μm and had a wide range of sizes. This work has been discredited since: 1) no one has been able to confirm the isolation of circular DNA from Ad 2; 2) the length of the DNA molecules was highly variable; 3) the length of the circular DNA was only about one-fourth the length of the linear Ad 2 DNA described by others.

Table 5

Physical Characteristics of Adenovirus DNA[a]

DNA from:	Buoyant density[b]	$S_{20,w}$		Length (μm)	Molecular weight (x 10^6 Daltons)
		Native	Denatured		
Ad 2	1.716	31.1	34.1	12.6 ± 0.8	24.4
Ad 4	1.717	31.0	34.7	11.6 ± 0.4	22.3 - 24.8
Ad 7	1.713	-	-	11.8 ± 0.8	22.7
Ad 12	1.708	30.6	33.5	11.0 ± 1.0	21.1 - 24.6
Ad 18	1.708	29.4	33.1	11.4 ± 0.5	21.9 - 22.8
Ad 21	1.714	-	-	13.0 ± 2.0	25.0

[a] Green et al. (1967b).

[b] Piña and Green (1964).

Table 6

Correlation Between Oncogenicity and Guanine plus Cytosine Content of Several Adenovirus DNA's[a]

Group	Antigenic type	Degree of oncogenicity	% DNA	Buoyant density of DNA	G+C from buoyant density	T_m	G+C from T_m
A	12	high	11.6-12.1	1.708	49	89.5	49
	18	high	11.6-12.1	1.708	49	88.8	47
	31	high	11.6-12.1	1.708	49	89.4	49
B	3	weak	12.6-13.0	1.714	55	90.3	51
	7	weak	12.1-12.6	1.713	54	90.3	51
	11	weak	12.6-13.0	1.712	53	90.0	50
	14	weak	12.6-13.0	1.715	56	91.0	52
	16	weak	12.6-13.0	1.714	55	90.0	52
	21	weak	13.0-13.5	1.714	55	90.8	52
C	1	nononcogenic	12.6-13.0	1.718	59	92.8	58
	2	nononcogenic	13.0-13.5	1.716	57	92.5	57
	5	nononcogenic	12.6-13.0	1.717	58	92.6	57
	6	nononcogenic	12.6-13.0	1.718	59	93.6	59

[a] Green et al. (1967c).

G + C = Guanine plus Cytosine.

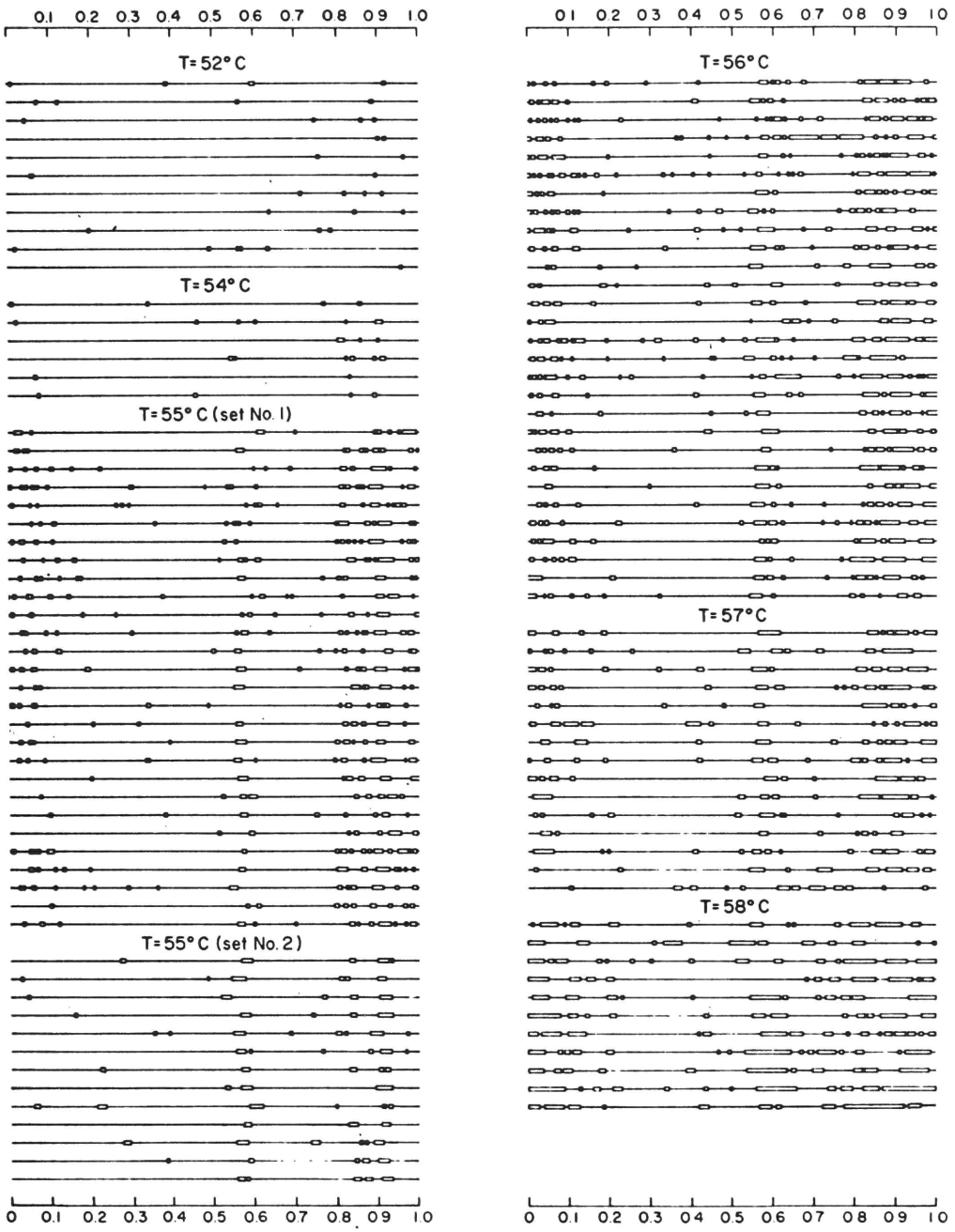

Figure 2: Thermal denaturation map of adenovirus type 2 DNA. The DNA was heated to the indicated temperatures in buffer containing 12% formaldehyde. The molecules were then examined in the electron microscope. The rectangles represent denatured regions and the lines represent native DNA. Each line represents a single oriented molecule. Figure 2 was taken from Doerfler and Kleinschmidt (1970).

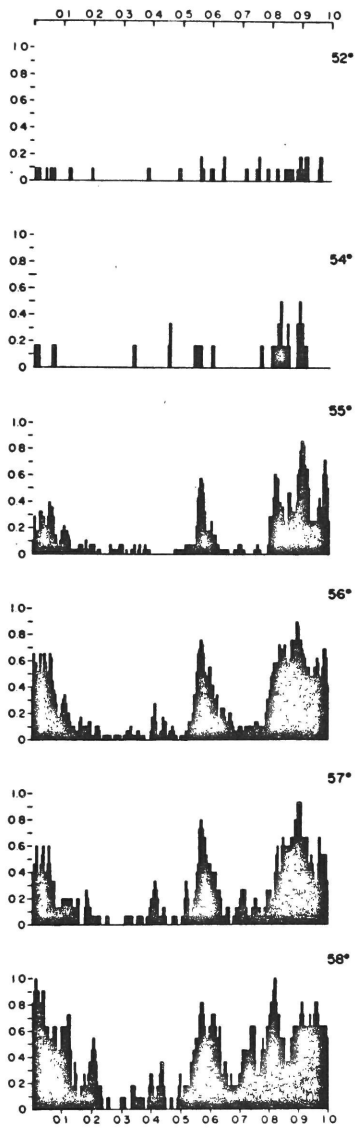

Figure 3. Frequency distribution of the thermal denaturation data illustrated in Figure 2. Thermal denaturation at 0.25, 0.50, and 0.70 the length of the Ad 2 DNA is relatively infrequent and occurs at higher temperatures. Thus these regions are presumably rich in guanine-cytosine base pairs. Figure 3 was taken from Doerfler and Kleinschmidt (1970).

D. Growth Cycle of Adenoviruses

Adenoviruses can have two types of interaction with cells. Cells can be productively infected and progeny particles synthesized. A one step growth curve is illustrated in Figure 4. The latent period is about 14 hr and the maximum titer of infectious virus is found about 48 hr after infection. At 36 hr after infection 4,000 to 10,000 PFU of virus per cell were newly formed, and 98% of the virus was still intracellular. Adenoviruses can also nonproductively or abortively infect cells. In this case, the adenoviruses produce little or no progeny, but certain virus-coded proteins are synthesized and some of the cells can be transformed. The nonproductive infection will be discussed in a later section (I. E.), in the context of oncogenesis and transformation.

The productive growth cycle can be divided into six steps: 1) adsorption, 2) penetration, 3) uncoating, 4) synthesis, 5) assembly and 6) release. Each of these steps will be considered in detail. The interpretation of all studies is limited since a high proportion of noninfectious particles (about 95%) is present in the inoculum.

1. Adsorption. The growth cycle begins with adsorption of the adenovirus particles to the host cells. For cells in monolayer culture, Ginsberg (1958) reported maximal adsorption of adenovirus types 1 to 4 within 4 to 6 hr. However, Green and Daesch (1961), using suspension cultures, found that adsorption was complete after 2 to 4 hr. However, in either case, little additional adsorption was observed after 2 hr. The adsorption period for adenoviruses is longer than that found for most other viruses. The adsorption period for the adenoviruses does not vary with the serotype.

Levine and Ginsberg (1967) demonstrated that the fiber of Ad 5 inhibited multiplication of Ad 5 by reducing the number of virions adsorbed to the KB cells. The fibers did not induce interferon. The virions taken up by the KB cells released their DNA normally but the biosyntheses of DNA, RNA and protein were blocked 20 to 25 hr after the addition of fiber antigen to cell cultures. Poliovirus synthesis was also inhibited in cells treated with Ad 5 fibers, but not by actinomycin D.

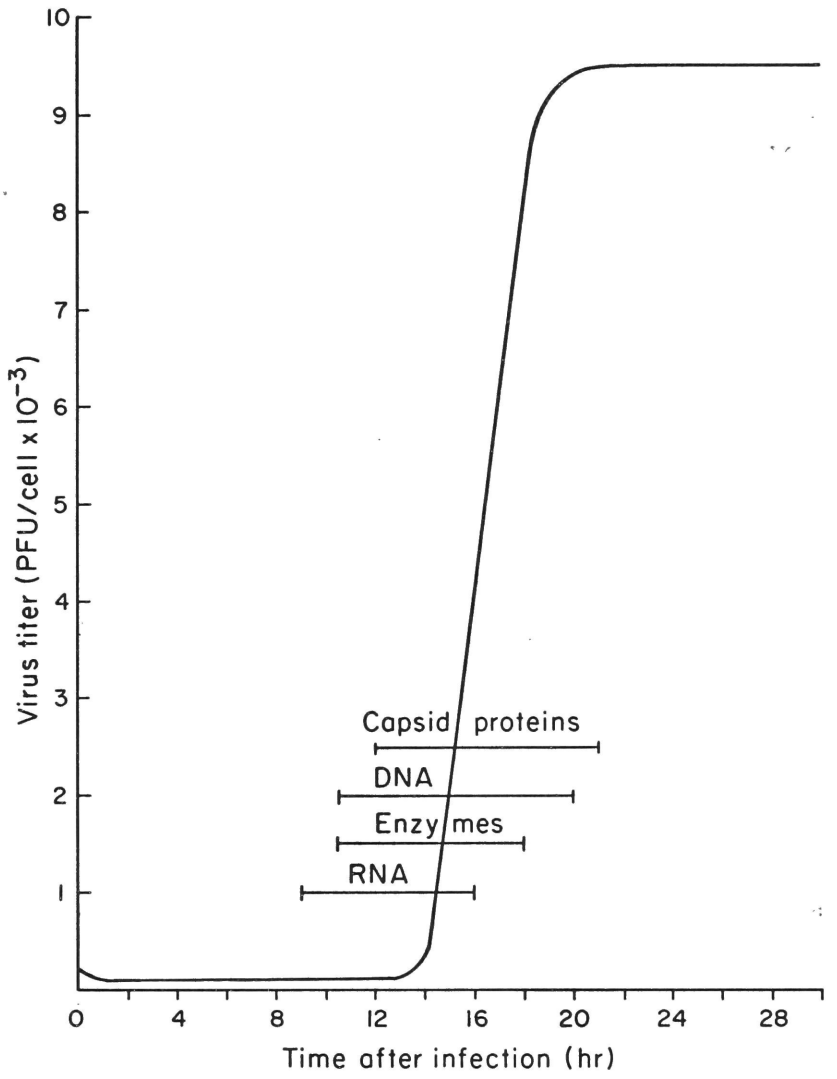

Figure 4: One step growth curve of adenovirus type 5. Adenovirus type 5 was used to infect KB cells at a multiplicity of 30 infectious particles per cell. Other types of adenovirus which productively infect cells have similar growth curves although the number of virus particles synthesized per cell and the onset of synthesis of viral macromolecules may vary. Figure 4 was modified from Ginsberg and Dingle (1965).

Philipson et al. (1968) established that Ad 2 adsorbs to specific sites on the plasma membrane of KB or HeLa cells. Each cell has about 10,000 receptor sites for Ad 2. These receptor sites were inactivated by pronase or subtilisin digestion. The receptor activity of enzyme treated cells started to return after 4 hr in growth medium and was normal after 8 hr. The receptor did not regenerate when protein synthesis was inhibited and thus the new receptors were probably synthesized and not replaced by preformed receptors.

Philipson et al. (1968) used purified hexons and fibers in competition studies, and showed that the Ad 2 fibers specifically inhibited virus attachment to the cell receptors. Inhibition required two fibers per receptor site. Adsorption of poliovirus was not affected by treating the cells with adenovirus fibers. The adenovirus hexons did not attach to the cells. Antiserum to fibers completely prevented the attachment of the fiber. Purified fibers of Ad 2 inhibited the adsorption of Ad 5. This cross inhibition indicated that the adenoviruses in subgroup 3 have common receptor sites.

Chardonnet and Dales (1970a,b) showed that adsorbed adenovirions are not in direct contact with the plasma membrane but remain at a distance of about the length of the fiber from the membrane. However, the fibers could not be visualized in electron micrographs. These data suggest that adsorption of the adenovirion to the cell surface is due to an interaction between specific protein containing receptors on the cell membrane and the fibers of the virion. Probably two fibers of the adenovirion interact with each cell receptor.

2. Penetration. Penetration of adenovirus particles into cells and uncoating of the virion are intimately related processes and have been studied in several laboratories by electron microscopy and/or physical and chemical techniques. However, the mechanism and events involved in penetration are still not clearly understood.

Morphological studies by Dales (1962) with Ad 7 suggested that adenovirus particles entered the cells by phagocytosis. Most of the Ad 7

was transported into lysosomes. However, Dales also found virus free in the cytoplasm. This raised the question whether a portion of the adsorbed virus might penetrate directly through the cell membrane or enter the cytoplasm from the vacuoles. Fong et al. (1968) studying a simian adenovirus, SV15, also have emphasized the role of phagocytosis in penetration.

Morgan et al. (1969) investigated penetration of purified Ad 7 into HeLa cells growing in monolayers. The initiation of entry was synchronized by adsorbing the Ad 7 to the cells at 4°C and then warming the complexes to 37°C. Lawrence and Ginsberg (1967) showed that adenoviruses adsorbed at 0°C but could not penetrate the cell until the temperature was increased. Philipson (1967) and Lawrence and Ginsberg (1967) had reported that the viral DNA became sensitive to deoxyribonuclease within 45 min after adsorption. Therefore, Morgan et al. investigated the events occurring within the first 45 min after shifting the temperature.

Morgan et al. (1969) found a wide variation in the number of Ad 7 particles adsorbed onto individual cells. Five minutes after the temperature shift, a few virions were observed in phagocytic vacuoles near the plasma membrane and the first free particles were found in the cytoplasm 10 min after warming the cells. Electron micrographs did not reveal any uncoating of virus in the phagocytic vacuoles.

Morgan et al. emphasized that release of Ad 7 from vacuoles only occurred 60 min or more after penetration. Nearly all of the virus in the cytoplasm was sequestered within vacuoles by 3 hr after penetration, and the numerous free particles which were present in the cytoplasm 1 hr after adsorption had disappeared. Ad 7 inactivated by formalin was adsorbed to a lesser extent and in this case all of the virions found inside the cells appeared within phagocytic vacuoles.

When phagocytosis was blocked with India ink or cellular metabolism was inhibited with colchicine, puromycin, dinitrophenol or arsenate, there was little effect on adsorption and no effect on the penetration of adenoviruses. Badly damaged cells did not contain free virus in the cytoplasm. Morgan et al. interpretation of these data is that the adenovirus enters

the cell both by direct penetration and by phagocytosis. Only those adenovirus particles which were free in the cytoplasm are uncoated, are transported to the nucleus and replicate the progeny virus.

Chardonnet and Dales (1970a) studied penetration of Ad 5 into HeLa cells. Their results indicated that after penetration adenovirions first appeared in cytoplasmic vesicles and were then released into the perinuclear cytoplasmic matrix. Viropexis was rapid and sometimes the particles were engulfed in vacuoles with a dense subjacent coat. Such vacuoles could develop from preexisting regions of cell membranes coated on the cytoplasmic side by dense, short elements. These vacuoles were found associated with viropexis of vesicular stomatitis virus by Simpson et al. (1969) and with the uptake of nonviral proteins by Roth and Porter (1962).

The kinetics of penetration are illustrated in Figure 5. These data suggested that the adenovirus particle was adsorbed, then entered into phagocytic vesicles and was released from the vesicles into the cytoplasm. Adenovirus particles within the lysosomes appeared to be blocked from replication. Incubation of the virus-cell complexes at different temperatures slowed the rate of penetration in proportion to the hypothermia employed. In electron micrographs, the adenovirus particles were found on the surface or in vacuoles and very few particles were near the nucleus. These data suggested that several stages of penetration were temperature-sensitive processes. Penetration out of the vacuoles was slower in stationary cell cultures than in suspension cultures.

Chardonnet and Dales (1970b) observed that adenoviruses in different groups showed differences in penetration. Adenovirus types 7 and 12 with short fibers were less firmly attached to the cells and were preferentially shunted into the lysosomes. Adenovirus types 1 and 5 with longer fibers were attached more firmly to the host cell and penetrated rapidly and in high proportion to the nucleus.

The physical and chemical studies of penetration are mainly concerned with the question of whether uncoating is a part of the penetration process. These methods are more sensitive and allow detection of small changes in

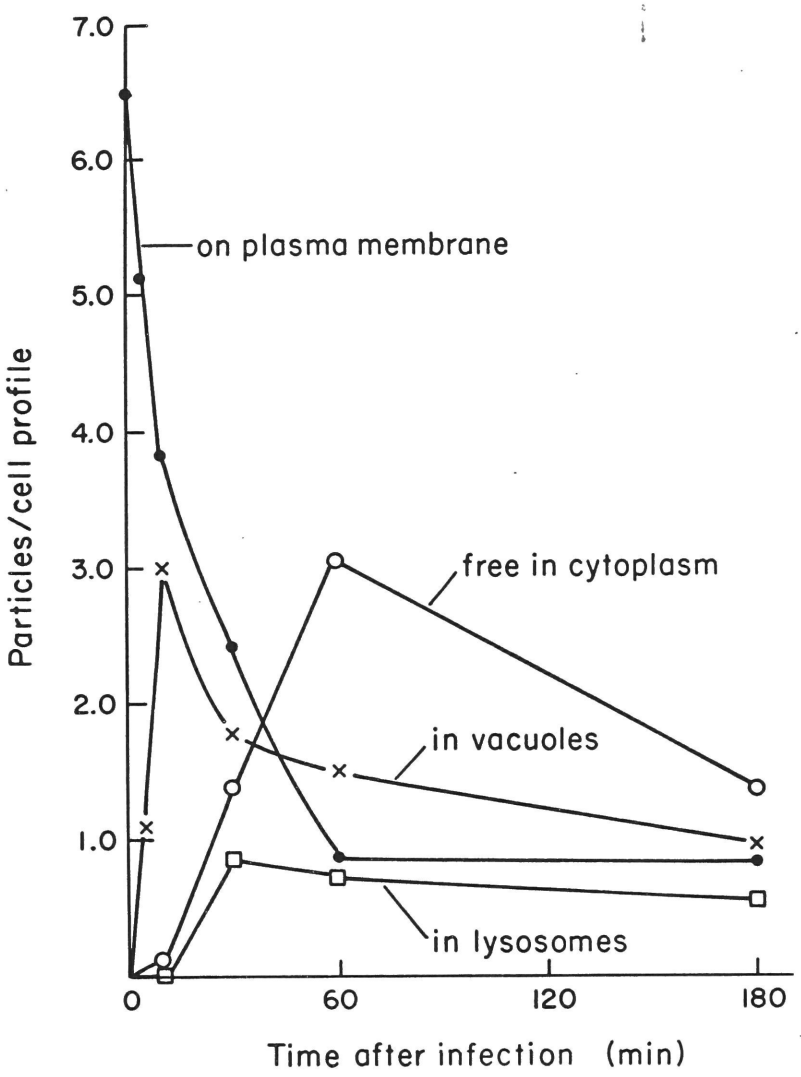

Figure 5: Kinetics of adenovirus type 5 penetration into HeLa cells. HeLa cells were inoculated with 10^3 Ad 5 particles per cell at 0°C. After unadsorbed virus was removed, the virus-cell complexes were incubated at 37°C, and sampled for electron microscopy at intervals. Cell-associated virus was enumerated on 100 to 200 cell profiles in each sample. Figure 5 was taken from Chardonnet and Dales (1970a).

the protein content of the virion. These studies will be included in the next section.

3. Uncoating. Uncoating is defined as the release of the viral nucleic acid from the virion. This process is operationally detected by determining the amount of viral DNA which becomes sensitive to hydrolysis by deoxyribonuclease. However, chemical studies demonstrated that the adenovirus DNA was sensitive to hydrolysis by deoxyribonuclease while most of the viral protein is still associated with the DNA (Russell et al., 1968).

The first signs of uncoating at the morphological level are subtle changes in the virus structure described by Morgan et al. (1969). The core of the virions within the cytoplasm had an increased granularity when compared to the core of Ad 7 virions found on the surface or within the phagocytic vacuoles. The surface of the capsid became less distinct, the crystalline faces were lost and the outline of the particle appeared spherical. This change in the particles free in the cytoplasm was obvious to Morgan et al. 10 min after penetration. In contradistinction, Chardonnet and Dales (1970a) found no morphological differences between Ad 5 particles adsorbed to the plasma membrane and those found free in the cytoplasm or within the nucleus.

Morgan et al. found that most of the adenovirus particles free in the cytoplasm ultimately laid close to the outer nuclear membrane. The association of the virus with nuclear pores seemed to be random. Within the nucleus, dense material resembling chromatin formed a pocket adjacent to the virion. The core of the virus contracted, the diffuse outer layer disappeared, and a sharply defined membrane-like structure was apparently applied to the nuclear membrane. The membrane-like viral structure proximal to the pocket appeared to rupture and the dense material of the viral core flowed into the nuclear pocket, leaving the empty viral membrane-like structure outside the nuclear membrane. The side of the nuclear pocket adjacent to the cytoplasm was closed off by a rim of chromatin and the nuclear and cytoplasmic membranes reformed to close the nuclear defect. The nuclear pocket then opened and the viral nucleic acid passed into the nuclear matrix. Some particles were discharged by a similar process through the nuclear pores.

Morgan et al., in contrast to Chardonnet and Dales (1970a), did not find any virions within the nuclei. Fong et al. (1968) have also found virions in electron micrographs of nuclei from SV15 (a simian adenovirus) infected cells. According to Morgan et al. the nucleoproteins of the adenovirus core appeared to be transported into the nucleus and apparently the capsid proteins remained in the membrane-like structure. These findings were not confirmed by the observations of Chardonnet and Dales (1970a,b), although physical and chemical data describing partially uncoated adenovirus particles agree with the morphology described by Morgan et al.

Uncoating has also been followed by physical and chemical studies in which the virus structures arising during the uncoating process are analyzed. Sussenbach (1967) showed that 1 hr after infecting HeLa cells with Ad 5, two types of particles were present: one type with a buoyant density of 1.34 g/cm^3 and another type with a buoyant density of 1.35 g/cm^3. The Ad 5 which had infected these cells had a buoyant density of 1.34 g/cm^3. When the infected cells were treated with trypsin before the cells were extracted and virus particles present in the cell extracts were analyzed, all of the particles with a density of 1.34 g/cm^3 were destroyed, whereas the particles with a density of 1.35 g/cm^3 were not affected. From these data, Sussenbach concluded that the adenovirus adsorbed to the cell surface was intact but that 1 hr after penetration into the cell the virions had lost some protein.

One hour after infection, 84% of the viral DNA could be hydrolyzed by deoxyribonuclease to acid soluble products although 85% of the viral proteins were present. Examination of these particles in the electron microscope revealed apparently normal adenovirions. These intracellular particles had low hemagglutination titers, were noninfective, and had lost about 5% of the protein found in intact particles. Sussenbach suggested that the pentons and fibers had been lost, and that this loss might have occurred during the process of adsorption.

Lawrence and Ginsberg (1967) studied uncoating of Ad 5 in KB cells and found that adsorption required 10 to 20 min, penetration took 30 to

60 min and uncoating had occurred within 60 to 90 min after infection. Uncoating proceeded normally when protein synthesis was blocked with puromycin but did not occur at 4°C. Neither viral DNA nor viral protein was degraded to acid-soluble material during the eclipse period. At 45 min after infection the Ad 5 still sedimented at 800 S, but partially uncoated forms which sedimented at 350 to 450 S were also present. The DNA of the slow sedimenting particles could be hydrolyzed by deoxyribonuclease. These partially uncoated particles were present until 4 hr after infection. Penetration was closely followed by uncoating, both processes occurred at similar rates and both were essentially complete by 60 min. Adenovirus type 5 which had been inactivated with ultraviolet light was uncoated at the same rate and to the same extent as control virus.

The kinetics of adsorption were similar regardless of whether the uptake of radioactivity or the disappearance of infectivity was measured. These data implied that all the viral particles, infectious and noninfectious, attached to cells similarly and that in this respect the viral population was homogeneous. The kinetics of the viral eclipse and the rendering of viral DNA sensitive to hydrolysis by deoxyribonuclease were alike. If the labeled infectious and noninfectious particles had not been similarly affected, the eclipse of infectivity would not have occurred at the same time as uncoating.

Philipson (1967) published a similar study of attachment, penetration and uncoating of Ad 2 in KB cells. Immediately after attachment, 5 to 8% of the virus protein was eluted from the cell. This virus protein had the size of free hexon subunits and reacted with antiserum prepared against hexons. Antiserum to other subunits was not tested.

Lonberg-Holm and Philipson (1969) extended the examination of uncoating of Ad 2 in HeLa and KB cells with physical techniques. Three intermediate forms of partially uncoated Ad 2 were isolated and partially characterized. There was a 4 to 5 min lag between adsorption and uncoating. The transition from attachment to the first partially uncoated intermediate had first order kinetics and the velocity constant was 0.065 min^{-1}. Thus the half-life for the adsorbed and uncoated virus was about 11 min. The

uncoating of the extranuclear virus was slow and increased steadily, while in the nuclear fraction, the amount of DNA sensitive to hydrolysis by deoxyribonuclease increased proportionally to the total viral DNA in the nucleus. At 30 min after infection, the particles were divided approximately equally between the nucleus and the cytoplasm. The uptake of virus into the nuclear fraction had about a 10 min lag period as compared to the cytoplasmic uptake. About 10% of the Ad 2 DNA taken up by the cells was degraded to acid-soluble products.

The first component containing labeled viral DNA which could be isolated was associated with membranes, had a density of less than 1.24 g/cm^3, was infectious when removed from the membranes by deoxycholate and its DNA was resistant to hydrolysis by deoxyribonuclease. Within 15 min after uptake into the cell, a second component was found which had a buoyant density of 1.35 g/cm^3 and its DNA could be hydrolyzed by deoxyribonuclease. After 15 min, one-half of the particles of the second component were associated with the cytoplasm and one-half with the nucleus. Nearly all of the virus particles associated with the nucleus had a density of 1.35 g/cm^3, but one-half of the particles in the cytoplasm had the density of complete Ad 2 virions. Analysis by zonal sedimentation of the second component indicated that it had nearly the same mass as complete virus particles. A third component was bound to a membrane and had a buoyant density of 1.29 g/cm^3. Treatment with detergent increased the density to 1.35 g/cm^3. However, there was a distribution of particles with a density between 1.2 and 1.35 g/cm^3. The sedimentation coefficient of the third component was about one-half the sedimentation coefficient of the intact virus. This component contained only 33% of the arginine found in the original virus. Virus cores had a density of 1.44-1.47 g/cm^3 but when isolated cores were mixed with cell extracts, the density decreased to 1.36 g/cm^3. Thus cell proteins were probably adsorbed. The third component was almost entirely associated with the nucleus. Most of the free Ad 2 DNA was found in the nucleus.

From these data an overall pattern can be recognized: Thousands of adenovirus particles can attach to each cell in less than 30 min. These

particles are altered to a partially uncoated product within 15 min, which is free inside the cell. There is no evidence that any intact virus which remains is refractory to uncoating. Although this fraction may represent intravesicular virions, it must also contain virus which is attached to nonviable cells or cell fragments. The partially uncoated particles are rapidly transformed to cores bound to cellular membranes and the free viral DNA is released into the nucleus.

4. Synthesis. The adenovirus genome of 20-25 million Daltons could code for about 100 proteins, each with a molecular weight of 100,000 Daltons. The 9 proteins of the virion and the DNA must be replicated to synthesize new infectious virus. In addition, the genome could code for many non-structural proteins. The biosynthesis of Ad 2, 4, and 5 have been studied in great detail and the sequence of synthesis which occurs is: a) RNA, b) early proteins or enzymes, c) DNA, and d) late proteins or capsid structural proteins. Finally, the DNA and structural proteins are assembled and infectious virus reappears. The presence of the virus products also has a profound effect on the cellular metabolism and this will be considered under e) effect on host cell metabolism.

a. RNA. The earliest biochemical event detected in the multiplication cycle of Ad 5 was the biosynthesis of RNA about 9 hr after infection (Flanagan and Ginsberg, 1964). The rate of RNA synthesis continued to increase until 13 to 14 hr after infection and then decreased to a level only slightly greater than that found in uninfected cells. The RNA synthesized was not a precursor of viral subunits but was clearly required for the production of infectious virus. It was likely that the major species of RNA made was messenger RNA.

Mak and Green (1968) studied the transcription of virus specific RNA in KB cells productively infected with Ad 12. Viral DNA synthesis began at 12 to 15 hr after infection. The rate of RNA synthesis in infected cells was stimulated 1.6 times when compared with uninfected cells and reached its maximum 20 hr after infection. Early viral mRNA was 0.1% of the RNA synthesized at 45 hr after infection. The mRNA found late in infection had been transcribed from the entire viral genome.

Mak (1969) reports that Ad 2 RNA synthesis began at 9 hr after infection and reached a peak value at 15 to 16 hr, but Ad 12 RNA synthesis began 12 to 13 hr after infection and peaked at 22 to 24 hr after infection. When both viruses coinfect cells, the RNA synthesis of Ad 2 and 12 occurred simultaneously, both starting at 9 hr, and reached their maximum at 20 hr. However, the amount of Ad 2 RNA synthesized was reduced 80 to 85%. Thus the Ad 2 genome advanced the synthesis of Ad 12 RNA, but the Ad 12 genome repressed transcription of Ad 2 DNA. The yield of infectious Ad 12 virus in coinfected cells was normal but the yield of infectious Ad 2 virus was markedly reduced. The Ad 2 might have provided early proteins for the transcription of Ad 12 as suggested by Chany and Brailovsky (1967) or Ad 2 infection might have affected the host cell metabolism and allowed Ad 12 to develop earlier than normal.

Ohe and Weissman (1970) isolated and analyzed the sequence of a stable, low molecular weight RNA from Ad 2 infected KB cells. They postulated that this 5 S RNA was an Ad 2 specific tRNA. However, this RNA annealed to both viral and cellular DNA. Ohe and Weissman assumed that the 5 S RNA might have contained regions of homology to cellular tRNA. There were no methylated or unusual residues present in this RNA. Ráska et al. (1970) could find no alteration of the tRNA fraction in KB or BHK cells infected with Ad 2.

b. Enzymes. The enzymes involved in nucleic acid biosynthesis have been studied most. Consigli and Ginsberg (1964a) demonstrated that between 11 and 18 hr after infection of HeLa cells with Ad 5, aspartate carbamyl transferase was increased two- to three-fold. However, this increase represented an increased synthesis of a cellular enzyme. Aspartate carbamyl transferase was not increased if Ad 5 DNA synthesis was inhibited. Consigli and Ginsberg (1964b) also found that thymidine kinase was elevated but the origin of the enzyme was not determined.

Ledinko (1966) found that monolayers of primary cultures of rhesus monkey kidney cells had one-fifth the thymidine kinase activity of continuous cell lines. Ad 2 was productive in monkey kidney cells but only 20 to 40 PFU/cell were formed while in KB cells 10,000 PFU/cell were produced.

Also, maximal viral synthesis in monkey cells was delayed until 50 hr after infection, when maximal viral synthesis usually occurred at 21 hr after infection in human cells. In monkey kidney cells Ad 2 assembly was delayed until 30 hr after infection but in KB cells assembly started at 13 hr after infection. Ninety-eight percent of the monkey kidney cells were infected and would no longer divide, but only 52 to 60% of the cells produced Ad 2. Extracts of monkey kidney cells infected with Ad 2 showed an elevated thymidine kinase activity. The specific activity of the enzyme started to increase at 15 hr, reached a maximum value at 50 hr after infection and then dropped to control levels by 100 hr. The specific activity of the thymidine kinase at 50 hr after infection was about 6 times the specific activity of uninfected control cells.

The thymidine kinase from infected cells had the same thermostability as the kinase of the uninfected control. However, the enzyme from infected cells was more sensitive to inhibition by deoxythymidine triphosphate and deoxythymidine diphosphate. The increase in thymidine kinase activity was inhibited by puromycin in Ad 2 infected cells. Thus there was de novo synthesis of thymidine kinase. The monkey kidney cells also had an increased deoxycytidine monophosphate deaminase, and aspartate carbamyl transferase at the time of maximum virus assembly. Ledinko (1968) found that human embryonic kidney cells infected with Ad 2 and Ad 12 have a four- to six-fold increase in thymidine kinase at 20 to 50 hr after infection but the deoxycytidine monophosphate deaminase was not elevated.

Bresnick and Rapp (1968) examined the thymidine kinase activity in primary cultures of kidney cells from African green monkeys. Human adenoviruses underwent an abortive cycle in simian cells which was characterized by the synthesis of virus specific T-antigen and viral DNA. However, capsid protein and infectious virus were not produced. Coinfection with SV40 or the simian adenoviruses SV15 or SA 7 allowed the human adenoviruses to finish their replication cycle.

Bresnick and Rapp found that thymidine kinase activity was increased in the productive cycle of Ad 2, 5, and 7 in human embryonic kidney cells and KB cells. Abortively infected simian cells had significantly elevated

thymidine kinase activity and when the helper virus was added, there was little or no further increase in thymidine kinase activity. Thus the early enzymes were synthesized normally in these abortively infected cells and the block in viral replication probably was due to the absence of late protein synthesis. Takahashi et al. (1966) found similar results in cells from newborn hamsters which were abortively infected with Ad 12 or were productively infected with Ad 5.

Kit et al. (1970) demonstrated that Ad 2, 4, 7, and 12 fail to induce thymidine kinase activity in HeLa cells deficient in kinase. The replication of Ad 5 was inhibited by hypoxanthine-aminopterin-thymidine-glycine in the kinase-deficient HeLa cells but not in the kinase-positive KB cells. These data further support the conclusion that adenoviruses do not code for a virus-specific thymidine kinase.

Green (1962) and Green et al. (1964) did not find any enhancement of DNA polymerase in Ad 2 infected KB cells. Similarly, Ledinko (1966) found no change in the DNA polymerase activity in rhesus monkey kidney cells infected with Ad 2.

However, Ledinko (1968) demonstrated that human embryonic kidney cells infected with Ad 2 and Ad 12 showed a two- to three-fold increase in DNA polymerase activity. The increase in activity appeared when the first new virus was synthesized and reached a maximum about 20 to 50 hr after infection and declined thereafter.

The partially purified DNA polymerase from Ad 2 or Ad 12 infected human embryonic kidney cells had the same saturation kinetics, and the same broad pH optimum as the cellular DNA polymerase. Extracts from infected cells did not activate DNA polymerase from noninfected cells. Extracts of noninfected cells did not alter the DNA polymerase activity of infected cell extracts. Puromycin inhibited the induction of DNA polymerase and arrested the increase when added after induction had begun. However, the induction occurred normally when 1 β-D-arabinofuranosylcytosine (Ara-C), an inhibitor of DNA synthesis, was added to the infected cultures. The increase in DNA polymerase activity was probably due to a cellular enzyme which was stimulated by the adenovirus infection.

Thus the activity of several specific cellular enzymes is increased in adenovirus infected cells. Virus coded enzymes have not been found. However, two proteins which are synthesized before the viral DNA is synthesized might be enzymes and one of these is clearly genetically determined by the adenovirus which infects the cell. These proteins are the T-antigen and the P-antigen. The significance of the T-antigen will be discussed later (Section I. E.). Also the T-antigen is present transiently in cells productively infected with adenovirus.

The P-antigen was first discovered by Russell et al.(1967a) as a protein induced in Ad 5 infected cells. The P-antigen does not cross-react with antisera against capsid proteins. Russell and Knight (1967) "aged" the Ad 5 capsids at 4°C until there were many broken capsids present when these particles were examined in the electron microscope. The P-antigen could be detected serologically in only the aged particles. Since the P-antigen was available only after partial degradation of the capsid, this protein was considered to be an internal structural protein of the core. The P-antigen was synthesized before DNA synthesis, but all other structural proteins were synthesized after DNA synthesis started. Russell and Knight suggested that the P-antigen was intimately related with the DNA and was partly responsible for the configuration of the DNA in the core.

Hayashi and Russell (1968) investigated the synthesis of viral antigens in Ad 5, 7, and 12 infected cells. The nuclear T-antigen was first synthesized at 7 hr after infection, it reached maximum concentration at 9 hr after infection, then it rapidly declined and disappeared by 13 hr after infection. The P-antigen appeared at 6 hr and reached a maximum at 7 hr after infection but only declined to about 50% of the maximum level. The capsid proteins were first synthesized at 10 to 12 hr after infection.

The P-antigens for Ad 5 and Ad 12 were specific and did not cross react. Thus at least the antigenic portion of this protein was determined by the viral genome. Russell and Becker (1968) reported that the production of the P-antigen was reduced in arginine deprived cells infected with Ad 5, while all of the capsid proteins were synthesized normally. Previously, Rouse et al. (1963) have shown that arginine deprived cells would not

replicate adenovirus. Russell and Becker suggested that this antigen or a component of it was a maturation factor similar to the one described for bacteriophage R17 by Roberts and Steitz (1967).

c. DNA. Biosynthesis of the adenovirus DNA began 10.5 to 11 hr after infection, about 4 hr before the first assembly of infectious virus particles (Ginsberg and Dixon, 1959; Green and Daesch, 1961; and Flanagan and Ginsberg, 1962).

Mak (1969) showed that Ad 2 DNA synthesis began at 10 hr after infection and peaked at 15 hr after infection. Ad 12 DNA synthesis started at 12 hr after infection and peaked at 20 hr. When Ad 2 and Ad 12 coinfected cells, the synthesis of Ad 2 DNA and Ad 12 DNA both started at 10 hr and peaked at 16 hr after infection, but the amount of Ad 2 DNA was reduced by about 90%. Again the Ad 2 DNA had supplied a helper function for the Ad 12.

d. Structural proteins. Wilcox and Ginsberg (1963a) showed that the structural protein subunits were synthesized 2 hr before production of mature virus. Russell et al. (1967a) found that the pentons were synthesized 2 hr after the hexons.

Hayashi and Russell (1968) have demonstrated that in human embryonic kidney cells infected with Ad 5, 7, and 12, the synthesis of hexon and fiber started about 10 hr after infection and the subunits reached a maximum concentration by 13 hr. But the synthesis of the penton base started about 12 hr after infection and the penton did not reach maximum concentration until 16 hr after infection.

Velicer and Ginsberg (1968) established that Ad 5 capsid proteins were synthesized in the cytoplasm and rapidly migrated into the nuclei of infected cells. Three independent methods demonstrated the cytoplasmic site of viral protein synthesis: 1) autoradiography showed that most if not all the proteins are synthesized in the cytoplasm and then transported rapidly into the nucleus; 2) immunological coprecipitation detected newly synthesized viral proteins in 200 S polyribosomes; 3) hybridization of denatured Ad 5 DNA with a species of RNA isolated from polyribosomes identified this RNA species as viral messenger RNA.

Velicer and Ginsberg (1970) studied the synthesis and transport of capsid proteins into the nucleus. At 20 to 24 hr after infection with Ad 5, 85% of the proteins synthesized by KB cells are virus specific. The virus proteins were synthesized on polyribosomes isolated from the cytoplasm with an average sedimentation coefficient of 200 S. The polypeptide chains synthesized during a 1 min period of labeling had an average sedimentation coefficient of 3.4 S in the presence of 1% SDS. The newly synthesized polypeptide chains were released from the polyribosomes within 1 min of completion and were transported into the nuclei within 6 min. During this 6 min interval, the single polypeptide chains assembled into polymeric proteins with average sedimentation coefficients of 6 S, 9 S, and 12 S. The 6 S and 9 S proteins were identified immunologically as fiber and hexon, respectively. The 9 S protein was tentatively identified as the penton base. Velicer and Ginsberg suggest that the subunits are assembled in the nucleus but there is little direct evidence to support this.

e. Effect on host cell metabolism. Ginsberg and Dixon (1959), Green (1962), and Polasa and Green (1967) have established that early after infection, the synthesis of DNA, RNA and protein by HeLa and KB cells continued at a normal rate. Ginsberg (1964) and Green et al. (1964) have shown that host DNA synthesis stops at approximately the time viral DNA synthesis begins. Green and Daesch (1961) and Bello and Ginsberg (1964) found that host protein synthesis is inhibited shortly after production of viral structural protein is initiated.

Ledinko (1966) has shown that the DNA, RNA, and protein synthesis of rhesus monkey kidney cells infected with Ad 2 is markedly inhibited. In this system the Ad 2 is not synthesized until 30 hr after infection. Protein and RNA synthesis were affected much more than DNA synthesis. The precursor pools were normal, but the RNA polymerase activity was reduced to 25% of normal by 24 hr after infection. At this time, less than 0.1% of progeny virus had been formed. Deoxyribonuclease activity was normal throughout the infection. The mechanism of the RNA polymerase inhibition was not elucidated.

Pereira (1960) demonstrated that HeLa cells infected with Ad 1, 2, 4, 5 and 6 produced a noninfectious factor which inhibited multiplication of Ad 5, poliovirus and vaccinia virus in HeLa cells. This inhibitor was neutralized by specific adenovirus antiserum to the fiber and the penton base.

Levine and Ginsberg (1967, 1968) reported that the fiber of Ad 5 inhibited synthesis of viral DNA, RNA and protein 20 to 25 hr after the pentons were added to the culture. The hexon did not attach to the cell and did not alter the cellular or viral metabolism. They found that hexons and fibers could bind to cellular or to Ad 5 DNA. The fibers and hexons were able to inhibit the DNA-dependent RNA polymerase and the DNA polymerase of KB cells. This inhibition could be overcome by increasing the concentration of DNA.

Fischer and Ginsberg (1957) noted that cells infected by adenovirus have an enhanced utilization of glucose and an increased accumulation of organic acids from carbohydrate metabolism. The increased glycolysis and the accumulation of organic acids by infected cells caused the media of infected cultures to become considerably more acid than those of uninfected cultures.

Bello and Ginsberg (1967) found that KB cells infected with Ad 5 had a normal rate of net protein synthesis but the net synthesis of host cell lactic dehydrogenase, acid phosphotase, deoxyribonuclease, fumerase, and phosphoglucose isomerase was stopped 16 to 20 hr after infection. This inhibition occurred shortly after the synthesis of capsid proteins began.

5. Assembly. The biosynthetic steps leading to the production of infectious adenovirus particles is a well-ordered, sequence of events. Despite the controlled orderliness of the process, it is not a highly efficient or economical one. Ginsberg and Dixon (1959) and Green (1962) showed that only 10 to 15% of the DNA and protein subunits synthesized in the infected cells became incorporated into infectious virus particles. Most polymers were synthesized in great excess and accumulated in the nucleus of the infected cell. These accumulations are the basis for the nuclear inclusions which form in infected cells. This has been clearly demonstrated by

Martinez-Palomo and Granboulan (1967) and Martinez-Palomo et al. (1967). These authors found four specific nuclear inclusions formed of: 1) only fibrillar viral DNA, 2) mainly capsid proteins, 3) protein and DNA aggregates, 4) dense reticular inclusions containing viral protein resistant to trypsin digestion but viral DNA susceptible to deoxyribonuclease.

The actual efficiency of assembly is genetically determined. Mak (1969) showed that Ad 2 and Ad 12 both synthesize the same amount of DNA and protein precursors but the yield of infectious particles for Ad 2 is ten times the yield for Ad 12. Coinfection did not alter the amount of Ad 12 assembled. Rainbow and Mak (1970) demonstrated a functional heterogeneity of virus particles in Ad 2 and Ad 12. Animal viruses generally have ratios of particles to infectious units greater than unity. For the human adenoviruses, these ratios vary from 11 to 23,000 for different types. Purified preparations of Ad 2 and Ad 12 have been found to contain defective particles capable of preventing a cell from cloning but unable to induce inclusion bodies or to form plaques. There are about ten times as many of these functionally defective particles in preparation of Ad 12 as in Ad 2. The mechanism of action of these defective particles in the cell is unknown. Errors in assembly may have produced these functionally defective particles.

The actual mechanism of adenovirus assembly from one molecule of DNA and the nine different proteins has not been studied. The assembly mechanism and intimate structure of the capsid subunits is only now being investigated. Warocquier et al. (1969) studied the thermosensitive block in Ad 2 infected KB cells. At 42°C no infectious virus is produced. However, early mRNA, late mRNA, DNA and presumably the early proteins are synthesized normally. The authors postulate that some step in the translation of late viral mRNA or assembly of the virion is blocked at the high temperature.

The hypothesis of self-assembly of virus postulated by Caspar and Klug (1962) is the central theory widely held. The assembly process is like crystallization in which two components of a simple virus will spontaneously combine to form virus particles since the assembled particle

represents their lowest energy state. This theory was formulated to explain the assembly of the simple helical viruses with identical subunits falling into identical positions where they maximize the number of bonds with their neighbors. However, the self-assembly of spherical viruses is not obvious since they often have multiple and nonidentical subunits and a closed spherical shell must be formed. Caspar and Klug suggested that this may be achieved by assuming quasiequivalence in which all chemical subunits, using the same bonds, are situated in approximately equivalent environments in an icosahedral lattice. As a consequence of this theory, it has to be postulated that the chemical subunits cluster into groups to maximize the number of bonds. These clusters or morphological units then give a particular virus its characteristic appearance in the electron microscope.

6. Release. Cells infected by adenovirus are not lysed to release the virus but continue active metabolism after virus synthesis, assembly and cytophatic changes have occurred. Ginsberg (1957) showed that cells with cytopathic changes, i.e. intranuclear inclusions, react with vital dyes as uninfected cells do up to 48 hr after infection. Ledinko (1966) showed that less than 20% of the Ad 2 synthesized by monkey kidney cells is released at 50 hr after infection.

The steps in the growth cycle of adenovirus are summarized in Figure 6.

E. Oncogenesis and Transformation

1. Tumor production in animals. The oncogenic potential of certain adenoviruses was first recognized when Trentin et al. (1962) showed that Ad 12 produced sarcomas when inoculated into newborn hamsters. Intensive work in several laboratories has shown that eight human adenoviruses, an avian adenovirus and six simian adenoviruses are oncogenic in baby hamsters. These data were reviewed by Huebner (1967). The capacity to produce tumors varies, as judged by the latent period and the minimum effective dose. Types 12, 18, and 31 have the highest oncogenic potential; Ad 12 has been most extensively studied. Adenovirus type 12 has also been shown to be

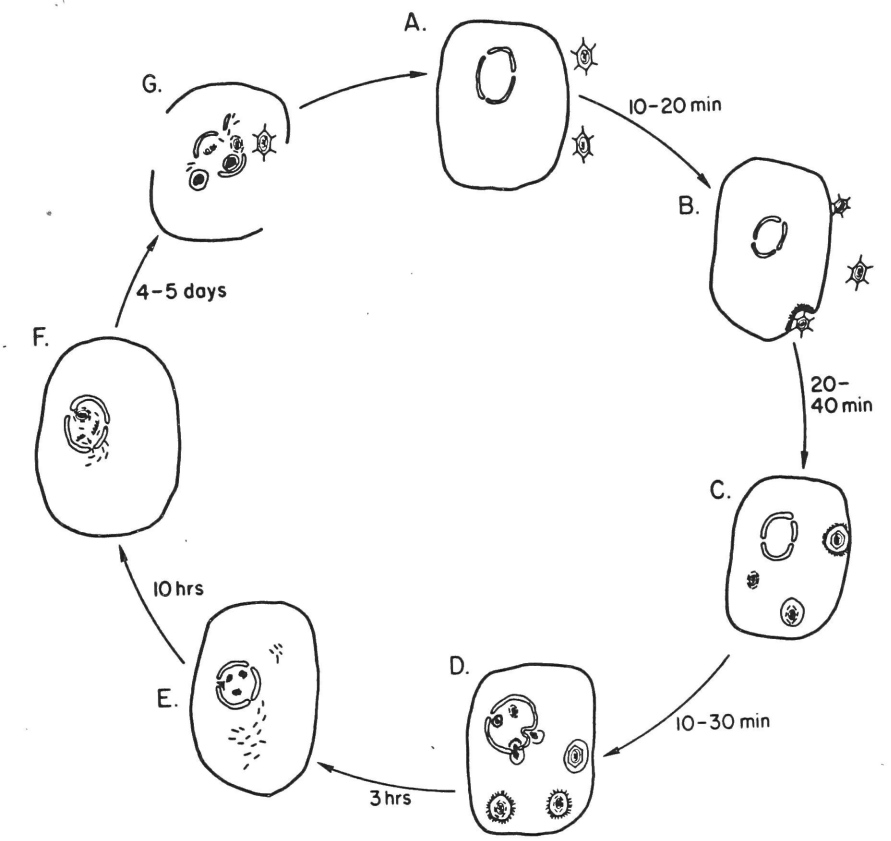

Figure 6: Steps in the life cycle of adenovirus. A. Infection of cells by adenovirus. B. Adsorption of adenovirions to the cells. C. Penetration of adenovirions and early events of uncoating. D. Uncoating of the adenovirus DNA and transport of the DNA into the nucleus. E. Synthesis of early RNA, P- and T-antigens. F. Synthesis of adenovirus DNA and structural proteins, assembly of first particles. G. Death of the host cell.

oncogenic for newborn rats by Huebner et al. (1963), the African rodent Mastomys by Rabson et al. (1964), certain strains of mice by Rabson et al. (1964) and Yabe et al. (1964).

Trentin's earlier observations, since extended by Kitamura et al. (1964), that no infectious adenovirus can be extracted from the tumors induced in hamsters by oncogenic adenoviruses, even after extensive treatment with chemical and physical inducing agents, have been confirmed by Huebner et al. (1962) and Landau et al. (1966).

2. Transformation studies in cell culture.

a. Alteration of cell biology. Most investigations with oncogenic adenoviruses have been performed in newborn hamsters or with cultured cells derived from tumors induced in newborn hamsters. However, McBride and Wiener (1964) demonstrated in vitro transformation of cultures of kidney cells from newborn hamsters after an incubation period of 8 to 10 weeks.

Freeman et al. (1967a) have described a more rapid and reproducible system of transformation by Ad 12 of rat embryo fibroblasts maintained in medium with low calcium concentration. Foci of transformed cells could be recognized 3 to 5 weeks after inoculation with the virus. Transformation followed one-hit kinetics, approximately 7×10^5 infectious particles were required for one transformation event. Cells transformed in vitro, like tumors produced by the virus in vivo, failed to yield infectious virus but they contain the specific adenovirus T-antigen which will be discussed later (Section I. E.2.b.3.).

Freeman et al. (1967b), using the rat embryo fibroblast system, have produced typical transformation with the group C, so-called "nononcogenic", adenovirus types 2 and 5. The T-antigen produced in Ad 2 transformed cells failed to cross react with T-antigens induced by group A or group B adenoviruses, and mRNA extracted from such cells hybridized with DNA of Ad 1, 2, or 5, but not with DNA from Ad 3, 4, or 12. The oncogenic potential of the group C adenoviruses in intact animals remains to be demonstrated.

Cooper et al. (1967) demonstrated that Ad 18 and Ad 4 induced chromosomal breaks in baby hamster kidney cells and in cultured human amnion cells. Both viruses halted mitosis of the cells. Chromosomal breaks were induced after mitosis was inhibited. The incidence of chromosome breaks was related directly to the input multiplicity. Stich and Yohn (1967) extended these findings to baby hamster kidney cells infected with Ad 12.

Zur Hausen (1967, 1968a) reported nonrandom chromosomal breaks in chromosomes 1 and 17 of human embryonic kidney cells 24 hr after infection with Ad 12. These chromosomal changes disappeared in persistently infected cultures. When the Ad 12 was inactivated by antiserum or UV irradiation, the number of chromosomal breaks was reduced. These data were interpreted to mean that viral DNA was associated with the cellular chromatin and certain chromosomal sites were preferred on chromosome 2. Zur Hausen (1968b,c) extended these findings to hamster cells infected with Ad 12 and found similar results but little tendency for the breakage of particular chromosomes. Essentially only the cells which contained Ad 12 by autoradiography and contained T-antigen, had chromosomal breaks. Thus the adenoviruses can cause direct chromosomal breakage in infected cells.

Homma et al. (1968) have demonstrated that HeLa cells persistently infected with Ad 2 contain a complete viral genome in every cell. A specific karyotypic change is induced in F-, G-, and X-group chromosomes in all virus carrier cells and in productively infected HeLa cells. Ebina et al. (1969a,b) demonstrated that one of the late-replicating X- and one of the G-21 chromosomes are missing and that two late-replicating metacentric chromosomes are added simultaneously to the F-group chromosomes.

Doerfler (1969) demonstrated that Ad 12 infected baby hamster kidney cells but did not replicate in these cells. Results were negative when techniques which permitted detection of Ad 12 DNA when it was more than 0.1% of the total intracellular DNA were used. The DNA synthesis of baby hamster kidney cells infected with Ad 12 appeared normal. However, when high multiplicities of infection were used, fragments of cellular DNA, five to ten million Daltons in molecular weight, were found 24 to 50 hr

after infection. These fragments may have been caused by an endonuclease cleaving the cellular DNA.

When baby hamster kidney cells were infected with Ad 2, virus was produced and synthesis of cellular DNA was inhibited about 17 hr after infection. If baby hamster kidney cells were infected with both Ad 2 and Ad 12, only Ad 2 DNA was synthesized. Thus, there was no complementation of the Ad 12 by Ad 2. The abortive infection of baby hamster kidney cells by Ad 12 must be specifically blocked in an early enzyme step since blockage occurs before DNA synthesis occurs.

b. Virus-coded antigens in transformed cells. The adenoviruses are the largest and most complex viruses which produce tumors in hamsters. Their DNA, with a molecular weight of 20 to 25 million Daltons, could code for about 100 "average" size proteins. Although the adenovirus transformed cells do not contain or release infectious virus, these cells do produce several virus-coded antigens. Three types of antigens have been recognized: 1) virion antigens, 2) transplantation antigen, and 3) T-antigen.

1) Virion antigens. Adenovirus type 12, in contrast to the other oncogenic adenoviruses, produces one and possibly two virion antigens in transformed hamster cells. The fiber antigen has been recognized by both complement fixation by Huebner et al. (1964) and gel diffusion tests by Berman and Rowe (1965). The gel diffusion tests revealed another antigen, "D", which reacts with human convalescent serum. When Pope and Rowe (1964) used specific immunofluorescent staining, fiber antigen was not recognized in individual cells. However, the concentration of fiber may have been too low, or as Strohl et al. (1966) suggested, only a small portion of the cells synthesized the fiber antigen at one time.

2) Tumor specific transplantation antigen. Trentin and Bryan (1966) demonstrated that both hamsters and mice could be protected against Ad 12 tumor cell transplants by preimmunization of adult animals with Ad 12. The authors concluded that tumor cells contained a novel virus specific transplantation antigen. The specificity of the tumor specific transplantation antigen of Ad 12 was investigated by Sjögren et al. (1967). Mice

infected with oncogenic Ad 7, 12, and 18 produced resistance to Ad 12 induced mouse tumor cells; thus there was at least partial cross reactivity between tumor specific transplantation antigens induced by these three oncogenic adenoviruses. However, mice infected with the nononcogenic Ad 5 did not produce resistance to the Ad 12 tumor cells.

3) T-antigen. The T-antigen, recently reviewed by Huebner (1967), is the best studied antigen associated with adenovirus tumor cells. The T-antigen occurred transiently in cells undergoing a cytolytic infection. Immunofluorescent tests demonstrated that the T-antigen was always present in transformed cells and persisted in these cells over many generations of cultivation in vitro. T-antigen appeared early in the cytolytic infections and synthesis of the T-antigen did not depend on prior synthesis of viral DNA.

The T-antigen of adenovirus apparently consists of two components which differ in heat stability and other properties (Huebner and Hollingshead, 1966). Complement fixation tests by Huebner et al. (1965) with sera from hamsters bearing adenovirus-induced tumors showed that the T-antigens fell into two cross reacting groups; those produced by Ad 3 and 7 and those produced by Ad 12 and 18. Subsequently experiments with other oncogenic human adenoviruses in cytolytic infection and with tumors induced in hamsters extended these two groups (Huebner, 1967): Group A: Adenovirus types 12, 18, and 31, the highly oncogenic adenoviruses. Group B: Adenovirus types 3, 7, 11, 14, 16, and 21, the weakly oncogenic adenoviruses. Pope and Rowe (1964) showed that the T-antigen was the most abundant antigen in transformed cells.

Gilead and Ginsberg (1968a) have purified the T-antigen from KB cells infected with Ad 12 and from hamster tumor cells. Immunoelectrophoresis indicated that the T-antigen was a single antigenic species. The T-antigen from the productively infected KB cells was immunologically similar to the T-antigen from the hamster tumor cells. The immunologically active molecule had the ultraviolet adsorption spectrum characteristic of a protein and was 85% inactivated by trypsin. The purified antigen was a single molecular species with a $S_{20,w}$ of 2.58. The purified antigen was inactivated at temperatures above 40°C (Gilead and Ginsberg, 1968b).

Tockstein et al. (1968) purified the T-antigen from the isolated nuclear fraction of KB cells infected by Ad 12 and hamster embryo cells transformed by Ad 12. A 200- to 400-fold purification of T-antigen was attained. The T-antigen contained three antigenic components by sucrose gradient centrifugation and four antigenic components by polyacrylamide gel electrophoresis. The T-antigen did not stimulate DNA synthesis in contact inhibited 3T3 cells. Furthermore, the T-antigen did not have the enzymatic activity of thymidine kinase, DNA polymerase, RNA polymerase, ribonuclease, or deoxyribonuclease. Nor did the T-antigen alter thymidine kinase, DNA polymerase or the DNA synthesizing activity of cell free extracts, or the RNA polymerase activity in isolated nuclei. The antigenic similarity between T-antigens of one type of adenovirus produced in different types of cells suggested that this protein was coded for by the viral genome.

Hamster cells transformed by adenoviruses apparently do not contain the complete genome of the infecting virus in a state which can be rescued or induced (Landau et al., 1966). However, Inoué and Nishibe (1967) reported that extracts of tumor cells contained an agent which induced T-antigen formation in HeLa cells. However, every transformed cell appeared to carry a portion of the viral genome, since all cells produced T-antigens characteristic for the type of adenovirus responsible for the transformation.

Strohl et al. (1966) tested the susceptibility of tumor cell from hamsters infected with Ad 12 to superinfection with Ad 2. All of the superinfected cells produced virion antigens of Ad 2. The yield of infectious Ad 2 was very low, but the yield could be increased 100-fold by treatment with mitomycin C. Strohl et al. suggested that most cells showed superinfection immunity, and that small amounts of infectious virus was produced from cells which had been "induced" and were able to produce both Ad 12 fiber antigens and complete infectious Ad 12.

3. Physical and biochemical data on the state of the adenovirus genome in transformed cells. Direct evidence for the presence of the adenovirus genetic material in cultured cells derived from Ad 12 induced hamster tumors

comes from Green and his colleagues. Fujinaga and Green (1966) pulse labeled tumor cells with uridine-^{3}H and extracted the RNA from the polyribosomes. Two to five percent of this RNA fraction hybridized with Ad 12 DNA. Further experiments by Green et al. (1966) showed significant hybridization between the DNA isolated from Ad 12 transformed cells and synthetic RNA made on an Ad 12 DNA template. These results suggested that only 0.001% of the tumor cell DNA was complementary to the viral DNA. This DNA was transcribed at a very high rate.

Fujinaga and Green (1967a) showed that each of the virus-specific mRNA's isolated from tumor cells induced by the weakly oncogenic adenovirus types 3, 7, 14, and 16 hybridized with DNA of each of the six weakly oncogenic Ad types, but that it did not hybridize with DNA from the highly oncogenic Ad types 12 and 18.

Fujinaga and Green (1967b) have demonstrated that the adenoviruses in group A are closely related. There is 30-60% homology, as determined from the degree of cross-hybridization of mRNA isolated from polyribosomes of transformed cells to viral DNA. By the same criterion, there was less than 10% homology between the adenoviruses of group A and group B. However, DNA-DNA homology between the DNAs of adenovirus groups A and B was about 15 to 30% (Lacy and Green, 1967). Thus common viral genes were transcribed in tumor cells induced by group A adenovirus. The potentially common genes between group A and B adenoviruses apparently are not expressed in transformed or tumor cells.

Doerfler (1968) presented physical evidence for the integration of Ad 12 DNA into the genome of baby hamster kidney cells. Baby hamster kidney cells were grown in 5-bromodeoxyuridine to increase the density of the cellular DNA. The dense cellular DNA was separable from the viral DNA by equilibrium sedimentation in CsCl density gradients. After the cells which contained dense DNA were infected with Ad 12 labeled with thymidine-^{3}H, some of the label was found associated with the cellular DNA in both neutral and alkaline gradients. This viral DNA which was associated with the cellular DNA remained associated with the cell but the viral DNA cosedimenting with the marker DNA decreased and eventually disappeared. When the

cellular DNA which contained the labeled viral DNA was fragmented, a labeled DNA species with a density intermediate between that of viral and cellular DNA was released. This later species of DNA probably contained one strand of viral DNA derived from the input virus and a newly synthesized dense strand of viral DNA. When the cellular DNA which contained labeled viral DNA was sedimented in alkaline and neutral sucrose gradients, the label sedimented with the cellular DNA. All these data provide strong physical evidence for a covalent linkage between the Ad 12 genome and the cellular DNA of baby hamster kidney cells.

When the available data are considered, a tentative picture of the relationship between adenovirus DNA and cellular DNA in adenovirus tumor cells can be drawn. It may be postulated that the oncogenic adenoviruses possess DNA segments which are complementary to segments of the cellular DNA and that regions of viral and cellular DNA overlap in their guanine and cytosine content, despite the differences in their overall base compositions. The greater divergence of guanine and cytosine contenst of the cellular DNA and the DNA of the nononcogenic adenoviruses may exclude such complementary overlap. Although only a minute portion of the cellular DNA is complementary to viral DNA, integration apparently occurs in virus transformed cells. The integrated viral DNA then must be expressed with high efficiency.

F. Scope and Goals of this Research

The metabolism of adenovirus DNA in productively and nonproductively infected cells was investigated. Most of the experiments were concerned with the size and the fate of the adenovirus DNA within these cells. Particular attention was focused on small DNA fragments arising from the viral DNA during infection. The hypothesis that these small fragments were the product of an endonuclease reaction was examined. The origin and fate of these new fragments were studied. The endonuclease which forms these fragments was localized to the penton, a capsid subunit of the virion. The fragments of adenovirus DNA apparently are incorporated into the incomplete adenovirions and may contribute to their synthesis and function. The fragments of viral DNA may also be involved as an intermediate in integration of the adenovirus DNA into the host cell genome.

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II. MATERIALS

A. Cell Culture Supplies

1. Media. The complete composition of all media and solutions used for cell culture is listed in Appendix A. Cells in monolayers were grown in either standard Eagle's (1955a,b,c) medium or reinforced Eagle's medium described by Bablanian et al. (1965). Infectivity of adenovirus was assayed on monolayers of human embryonic kidney cells in secondary passage using the overlay described by Strohl et al. (1967). Suspension cultures were grown in spinner medium. Spinner medium is Eagle's (1959) medium without $CaCl_2$ enriched with 100 micromoles of each nonessential amino acid per liter.

2. Sera. Lipostabilized calf serum was purchased from Grand Island Biological Company, Grand Island, N. Y., or Microbiological Associates, Bethesda, Md. Fetal calf serum was purchased from Grand Island Biological Company.

3. Tryptose phosphate broth. Tryptose phosphate broth, used as a protein supplement, was a phosphate buffered saline containing dextrose and 2% tryptose (Difco). The composition of tryptose phosphate broth and the analysis of tryptose is included in Appendix A.

4. Trypsin-versene solution. Monolayers of cells were dispersed with trypsin-versene solution. This solution contained 0.25% trypsin and 0.05% EDTA in isotonic saline buffered with phosphate to pH 7.2.

5. Phosphate buffered saline. Phosphate buffered saline (PBS) was described by Dulbecco and Vogt (1954). This isotonic solution is buffered with phosphate to pH 7.2 and contains calcium, magnesium and antibiotics.

B. Cell Cultures

1. KB cell cultures. The KB cell line (CCL17) was obtained from the American Type Culture Collection. KB cells were originally isolated by Eagle (1955b) from a human epidermal carcinoma of the mouth and tongue. The KB cells were grown in suspension culture in spinner medium supplemented with 5% calf serum or in monolayers with Eagle's medium supplemented with 5% calf serum.

2. Baby hamster kidney cell cultures. Baby hamster kidney (BHK21) cells, clone 13 (BHK21/13), were given to us by Dr. Carabazzo of the Rockefeller Foundation Virus Laboratory. The BHK cells were originally isolated by Stoker and Macpherson (1964) from the kidneys of 1 day old Syrian hamsters. The BHK cells were grown as monolayers using reinforced Eagle's medium supplemented with 10% tryptose phosphate broth and 10% calf serum.

3. Human embryonic lung cell cultures. Human embryonic lung (HEL) cells, derived from a fetal lung digested with trypsin, were supplied by Dr. P. Choppin of The Rockefeller University. The HEL cells were grown as monolayers using reinforced Eagle's medium supplemented with 10% tryptose phosphate broth and 10% calf serum.

4. Human embryonic kidney cell cultures. Primary human embryonic kidney (HEK) cells were purchased from Microbiological Associates, Inc. and were grown in Eagle's medium supplemented with 5% calf serum.

5. HeLa cell cultures. The HeLa cell line, originally isolated by Gey (Scherer et al., 1953), from an epidermoid carcinoma of the human cervix, was supplied by Dr. B. Mandel of the Public Health Research Institute of the City of New York. The HeLa cells were grown in monolayers using Eagle's medium supplemented with 5% calf serum or in suspension culture with spinner medium supplemented with 7% calf serum.

Some characteristics of the cells described in this section are summarized in Table 7.

C. Preparation of Adenovirions and Adenovirus DNA

1. Strains of adenovirus. Preparations of human adenovirus type 12, strain Huie, were originally isolated in 1954 by Kilbrick et al. (1957) and were identified by Rowe et al. (1956) and were further described by Pereira et al. (1963). The original stock of adenovirus type 12 used in these studies was supplied by American Type Culture Collection and by Dr. Wallace Rowe of the National Institute of Allergy and Infectious Diseases. Adenovirus type 2, strain adenoid 6, originally identified by Huebner et al. (1954) and further described by Rowe et al. (1955), and Pereira et al. (1963) was obtained from the American Type Culture Collection.

Table 7

Characteristics of Cells Used in Adenovirus Experiments

Cell line	Morphology	Type of cell line	Generation time (hr)
KB	epithelial	continuous	24
HeLa	epithelial	continuous	24
BHK21/13	fibroblastic	continuous	24
HEL	fibroblastic	primary	72
HEK	epithelial	primary	36-48

The identity of each virus preparation was established from the buoyant density of the virus and the buoyant density and sedimentation coefficient of the DNA extracted from the purified virions.

2. Production of adenovirus. Adenovirus was produced in KB cells by a modification of the procedure described by Green and Piña (1963a). A suspension culture of KB cells was grown to a density of approximately 3×10^5 cells/ml in spinner media supplemented with 5% calf serum. The cells were sedimented by centrifugation and resuspended in one-twentieth of the original volume of medium warmed to 37°C. Adenovirus type 2 or 12 at a multiplicity of infection of about 100 PFU/cell was adsorbed for two hours at 37°C with constant stirring. Then the infected cells were diluted to a concentration of approximately 3.5×10^5 cells per ml with spinner media which contained 10% calf serum and maintained in suspension culture for 48 hr.

When the viral DNA was isotopically labeled, 1 μCi/ml of thymidine-6-^{3}H, 0.5 μCi/ml of formate-^{14}C or 2.0 μCi/ml of $^{32}PO_4$ was added to the culture immediately after adsorption. When the viral proteins were isotopically labeled, 1 μCi/ml of L-proline-^{3}H or of L-leucine-4,5-^{3}H was added to the culture 10 hr after infection. When L-proline was used, the nonessential amino acids were deleted from the spinner medium (see Appendix A).

About 48 hr after infection, the cells were pelleted by centrifugation and were resuspended in 5 ml of PBS. The cell suspension was placed in an ice bath and the cells were disrupted by a Branson sonifier operated at a power setting of 2.2 amp for 3 min. The homogenate which resulted was centrifuged for 10,000 g-min at 4°C, and the supernatant fluid was saved. The pelleted debris was resuspended in 1 ml PBS, and then sedimented by 15,000 g-min at 4°C. This supernatant fluid was combined with the first supernatant fluid and was stored at -60°C until the virus was purified.

When the viral DNA with orthophosphate-^{32}P was labeled, phosphate was deleted from the spinner medium. The division rate of KB cells grown in spinner medium without phosphate but supplemented with 10% calf serum

was determined. The concentration of cells was determined with a hemocytometer at 24 hr intervals and the culture was diluted with an equal volume of new medium. The data in Table 8 indicate that when the cells are deprived of phosphate for more than 3 days, cell division slowed or stopped.

Since the production of high yields of adenovirus in KB cells requires 48 hr and the KB cells deprived of phosphate divide nearly normally during this time, the yields of Ad 2 in 100 ml of KB cells growing in normal medium and medium without phosphate were compared. Thymidine-^{3}H or orthophosphate-^{32}P label was added to each culture and the amount of purified virus which was produced and the specific activity of the Ad 2 was determined. Table 9 shows the results of this experiment. When thymidine-6-^{3}H is used as the label, the phosphate deprived cells produce nearly the same amount of virus as the control cells, and the specific infectivity of the virions is the same. However, the specific activity of the Ad 2 is reduced by 45%. When the DNA is labeled with orthophosphate-^{32}P and phosphate was present in the medium, the amount of virus produced is reduced by 19% but when phosphate was deleted, the virus yield is reduced by 96%. When the medium contained phosphate, the specific activity of the Ad 2 labeled with ^{32}P is only 41% as high as the specific activity when thymidine-6-^{3}H was used. When the phosphate was deleted from the medium, the specific activity of the Ad 2 labeled with ^{32}P is 2.4 times higher than the specific activity of the Ad 2 produced in medium which contained phosphate and was labeled with thymidine-6-^{3}H. The specific infectivity of the virus labeled with ^{32}P is 400 times less than the specific infectivity of the virus labeled with thymidine.

The low yield and low specific infectivity of the Ad 2 labeled with ^{32}P were inversely correlated with the specific radioactivity of the Ad 2. The low yield and infectivity was probably caused by breakage of the nucleic acid strands during synthesis, assembly and storage by the recoil energy of the ^{32}P atoms which decayed. The low yields and low specific activity of Ad 2 labeled with ^{32}P and the short half life of ^{32}P

Table 8

Phosphate Requirement for Division of KB Cells

Day	KB cells/ml (x 10^{-5})[a]	
spinner medium:	with PO_4	without PO_4
0	8.9	10.2
1	9.5	9.8
2	8.0	8.9
3	7.5	3.8
4	7.3	2.0
5	6.8	1.2

[a] Concentration of KB cells present at the end of the 24 hr growth period. After the cells were counted the cell culture was diluted with an equal volume of spinner medium supplemented with 10% undialyzed calf serum.

Table 9

Phosphate Requirement for Production of Adenovirus Type 2

	Thymidine-^{3}H added (1 μCi/ml) Spinner medium		Phosphate-^{32}P added (2 μCi/ml) Spinner medium	
	with PO_4	without PO_4	with PO_4	without PO_4
A_{260} of purified virus (ODU)	1.32	1.22	1.06	.05
Dry weight of Ad 2[a] (μg)	655	610	530	24
Specific radioactivity[b] (dpm/μg)	300	163	124	720
Specific infectivity[c] (PFU/μg)	1.6×10^9	1.7×10^9	1.4×10^8	3.7×10^6

[a] The amount of purified Ad 2 recovered from 100 ml of infected KB cells. The volume and absorption at 260 nm of the Ad 2 was determined.

The dry weight of Ad 2 was estimated from these data with the method described by Warburg and Christian (1941).

[b] The amount of radioactivity (counts per minute - cpm) which had been incorporated into the purified Ad 2 was determined by liquid scintillation spectrometry. The values for disintegrations per minute (dpm) were calculated from the values for counts per minute. The calculations corrected for background, quenching, self-absorption and counting efficiency.

[c] The infectivity titer was determined for HEK cells.

restricted the use of the virus labeled with ^{32}P. Hence the adenoviruses were usually labeled with thymidine-^{3}H and formate-^{14}C. Also, the DNA labeled with ^{32}P rapidly accumulates single-strand breaks when the ^{32}P atoms disintegrate. The metabolism of fragmented Ad 2 DNA in infected cells was not investigated. However, the results of Lawrence and Ginsberg (1967) with UV inactivated Ad 5 suggest that the inactivated Ad 2 labeled with ^{32}P will at least be uncoated.

3. Purification of adenovirions. The adenovirus was purified by equilibrium sedimentation in CsCl density gradients as described by Green and Piña (1963a, 1964) and as modified by Doerfler (1968b, 1969). The clarified supernatant fluid from the homogenized infected cells was extracted twice with equal volumes of trichlorotrifluoroethane. The phases were mixed well, and were then separated by centrifugation at 4°C by 5000 g-min. The trichlorotrifluoroethane phase was withdrawn along with the interphase gel. The combined trichlorotrifluoroethane phases and interphase gels were mixed with 2 ml of PBS, and the phases separated by centrifugation. The aqueous phase was removed and combined with the aqueous phase derived from the homogenate.

A S40 centrifuge tube made of nitrocellulose was filled with 6.0 g CsCl, 1 ml glycerol and the extracted supernatant fluid which contained the adenovirus. The tube was filled to 13.5 ml with PBS and was centrifuged at -4°C at 35,000 rpm in the S40 rotor. A minimum of 900,000 g-hr was required for the CsCl gradient to reach equilibrium. Centrifugation at only 25,000 or 30,000 rpm decreased the slope of the CsCl gradient and thus enhanced the separation and purification of particles with similar densities. The bottom of the tube was punctured, and the various visible bands of virus particles were collected individually. The virus was purified further by equilibrium centrifugation in CsCl gradients three times. The purified particles could be stored for weeks in the isopycnic CsCl solution without any change in the physical characteristics of the virion although the infectivity titer declined by about 50% after one month. The virus was dialyzed five times against 1000 volumes of PBS at 4°C before the virus was used.

One liter of infected KB cells (4 x 10^8 cells) produced 5 to 15 mg of purified Ad 2 virions or 1 to 2 mg of purified Ad 12 virions. Green and Piña (1963a) reported yields of 5 to 15 mg of purified Ad 2 from 6 to 9 x 10^8 cells. Nearly 90% of the infectivity found in the supernatant fluid from the sonicated cells was recovered as purified virus.

The degree of purification of the virus was determined by an isotope dilution experiment. One liter of KB cells at 3.5 x 10^5 cells/ml which contained tritium labeled DNA or protein with a high specific activity, was added to one liter of KB cells at 3.5 x 10^5 cells/ml which were infected with Ad 2 and produced without label. Then the virus was purified and the amount of tritium associated with the particles during the purification was determined. The data from this experiment are listed in Tables 10 and 11. The mechanism of purification of the virus particles from cell protein or cell DNA was mainly by dilution of the low molecular weight contaminants out of the bands of purified Ad 2. The high molecular weight contaminants were removed by isopycnic sedimentation. Each microgram of purified Ad 2 could contain 0.01 ng KB cell protein and 0.2 ng KB cell DNA.

4. Preparation of adenovirus DNA. Viral DNA was prepared by the method described by Doerfler (1969), slightly modified in that the purified virus was dialyzed extensively against either TE buffer (TE buffer = 0.01 M tris-HCl buffer at pH 7.2 and 0.001 M EDTA) or PBS at 4°C. The dialyzed virus was stored at 4°C for about 10 days or until the virus precipitated. During this storage the virus became more susceptible to proteolytic digestion.

Table 12 lists the sedimentation coefficients during aging of Ad 2 virus labeled with proline-^{3}H. The marker used in these studies was Ad 2 labeled with formate-^{14}C. The marker was purified and immediately added directly from the CsCl solution to the sample. At least 90% of the tritium label placed on the gradient cosedimented with the marker. A few percent of the ^{3}H-label sedimented at 1240 S, especially after the Ad 2 had been stored for 3 to 4 days. The fast sedimenting material may represent Ad 2 aggregates. There is no change in sedimentation coefficient during the

Table 10

Purity of Adenovirions and Adenovirus DNA

Isotope dilution experiment using tritium-labeled KB-cell protein						
Step of purification	Volume (ml)	µg Ad 2[a]	A_{260}/A_{280}	cpm/ml	mg KB protein[b]	µg KB protein/ µg AD 2
Supernatant fluid of sonicated cells	6.0	1264[c]		982,248	49.3	390
Supernatant fluid after trichlorotrifluoroethane extraction	6.0	1264[c]		934,509	46.9	371
Supernatant fluid diluted before the first CsCl gradient	13.0	1264[c]		360,420	39.2	310
After first CsCl gradient:						
band of Ad 2 particles	1.5	1264	1.04	236,672	3.0	22.9
CsCl solution	11.5			487,734	36.9	
After second CsCl gradient:						
band of Ad 2 particles	1.15	580	1.26	31,880	0.3	0.5
CsCl solution	11.85			35,790	2.5	
After third CsCl gradient:						
band of Ad 2 particles	0.60	491	1.40	1,630	0.005	0.0001
CsCl solution	12.40			2,380	2.0	
Ad 2 after dialysis	0.40	278	1.41	656	0.0005	0.00001
Ad 2 DNA after extraction and dialysis	0.55	33[d]	1.98	72	0.000024	0.00073[e]

Table 11

Purity of Adenovirions and Adenovirus DNA

Isotope dilution experiment using tritium-labeled KB-cell DNA						
Step of purification	Volume (ml)	µg Ad 2[a]	A_{260}/A_{280}	cpm/ml	µg KB DNA[f]	µg KB DNA/ µg Ad 2
Supernatant fluid of sonicated cells	6.0	643[c]		3,999,930	2682	4.17
Supernatant fluid after trichlorotrifluoroethane extraction	6.0	643[c]		2,249,930	2514	3.91
Supernatant fluid diluted before the first CsCl gradient	13.0	643[c]		736,300	1069	1.66
After first CsCl gradient:						
band of Ad 2 particles	0.75	643	1.05	300,800	25.2	0.039
CsCl solution	12.25			382,248	923.3	
After second CsCl gradient:						
band of Ad 2 particles	1.45	394	1.31	9,890	1.6	0.004
CsCl solution	11.55			15,920	20.5	
After third CsCl gradient:						
band of Ad 2 particles	0.90	347	1.41	770	0.077	0.0002
CsCl solution	12.10			1,170	1.58	
Ad 2 after dialysis	0.60	194	1.41	590	0.039	0.0002
Ad 2 DNA after extraction and dialysis	0.80	23[d]	2.00	58	0.005	0.0002[e]

Footnotes for Table 10 and Table 11

[a] Estimated from A_{260}, (1 ODU_{260} = 206 µg Ad 2) large errors are made when impure samples are used.

[b] Determined from the specific activity of KB protein, 1 mg = 119,471 cpm.

[c] Assumed to be the minimum quantity present from the value determined in a later step.

[d] µg Ad 2 DNA.

[e] µg KB protein or µg KB DNA per µg Ad 2 DNA.

[f] Determined from the specific activity of KB DNA, 1 µg = 8,948 cpm.

Table 12

Sedimentation Coefficient of Proline-^{3}H Labeled Adenovirus Type 2 after Dialysis and Storage

Days after dialysis	Sedimentation coefficient
0	780
1	777
2	785
4	794
5	787
6	780
7	780
8	782
9	780

[a] The sedimentation coefficient determined by zone velocity sedimentation in 5 to 20% neutral sucrose gradients. The virus was sedimented for 40 min at 17,500 rpm in the SW 50.1 rotor. Fresh Ad 2 labeled with formate-^{14}C was used as an internal marker.

aging process and little or no proline label remains at the top of the gradient when the aged virus is sedimented.

One volume of pronase solution was incubated for 30 to 60 min at 37°C with ten volumes of precipitated or aged virus. The digested virus was extracted three times with equal volumes of phenol which had been saturated with PBS or TE buffer. The phenol extractions were followed by two extractions with equal volumes of diethyl ether. The DNA was then dialyzed for 24 hr against five changes of 1000 volumes of TE buffer or PBS.

More than 90% of the viral DNA was extracted by this method. When Ad 2 labeled with L-methionine-^{35}S was extracted, less than 0.02% of the label, and thus presumably less than 0.02% of the protein, remained associated with the viral DNA. The data in Tables 10 and 11 indicate that each microgram of DNA extracted from purified Ad 2 may contain 0.73 ng KB cell protein and 0.2 ng KB cell DNA.

5. Credentials of adenovirions and adenovirus DNA.

a. Quality of the adenovirions. Green and Piña (1963a) reported that more than 80% of the Ad 2 was recovered from the cell homogenate as purified virus by a similar purification scheme with RbCl gradients. The purity of the adenovirus was established by the isotope dilution experiment. The data in Tables 10 and 11 indicate the adenovirus particles may contain 0.001% cell proteins and 0.02% cell DNA. Green and Piña (1963a) determined by isotope dilution methods that 0.03% of the phosphorous associated with the purified virus was derived from KB cell nucleic acid. They also determined by quantitative complement fixation that less than 0.01% of the protein associated with the purified Ad 2 was KB cell protein. The Ad 2 purified for these experiments were contaminated with less KB cell protein and DNA than the preparations described by Green and Piña (1963a).

The physical parameters defining the Ad 2 and Ad 12 particles are listed in Table 13. The ultraviolet absorption of the virus in 4 M CsCl solutions in PBS was determined at 260 nm and 280 nm in a Zeiss PMQ II spectrophotometer with microcuvettes. The linear relationship between

Table 13

Physical Characteristics of Adenovirions and Adenovirus DNA

	Ad 2	Ad 2 DNA	Ad 12	Ad 12 DNA
A_{260}/A_{280}	1.31 ± 0.22[a]	1.98 ± 0.09	1.28 ± 0.27	2.01 ± 0.12
	N = 27	N = 16	N = 14	N = 9
Specific activity[b] when labeled with:				
a. Thymidine-^{3}H	6.59 ± 1.41	6.82 ± 0.67	7.41 ± 1.86	7.44 ± .76
	N = 18	N = 21	N = 12	N = 15
b. Formate-^{14}C	5.61 ± 1.17	2.90 ± 0.47	5.94 ± 1.34	2.99 ± 1.10
	N = 8	N = 8	N = 3	N = 3
c. Proline-^{3}H	2.44 ± 1.21	.74 ± 0.98	1.98	.58
	N = 6	N = 6	N = 1	N = 1
d. $^{32}PO_4^{\equiv}$	$6.2 \times 10^{3}{}_{2} \pm 1.3 \times 10^{2}$	$6.97 \times 10^{3} \pm 9 \times 10^{2}$	1.4×10^{2}	1.5×10^{3}
	N = 4	N = 4	N = 1	N = 1
Specific infectivity[c]	$4.3 \times 10^{7}{}_{7} \pm 1.9 \times 10^{7}$	-	$2.1 \times 10^{7}{}_{7} \pm 1.1 \times 10^{7}$	-
	N = 9		N = 11	
Buoyant density	1.3345 ± .00157	1.7127 ± .00147	1.3224 ± .00169	1.7048 ± .00181
	N = 57	N = 9	N = 24	N = 12
Sedimentation coefficient	831 ± 41	31.31 ± .118	791 ± 36	28.98 ± .39
	N = 6	N = 26	N = 4	N = 17

[a] Mean ± SEM

N = number of determinations

[b] nCi/micromole

[c] PFU/ng

the absorption at these two wavelengths and the concentration of the virus was determined over a range of 0.1 to 1.0 ODU, and is illustrated in Figure 7. The ratio of absorption at 260 nm to 280 nm is close to the value of 1.286 reported by Lawrence and Ginsberg (1967) for Ad 5 and 1.31 reported by Philipson (1967) for Ad 2. These values have not been corrected for scattering. Maizel et al. (1968a) and Lonberg-Holm and Philipson (1969) compared the absorption at 260 nm with intact Ad 2 and Ad 2 dissolved in SDS and found light scattering was less than 10% of the absorption. The data in Figure 7 indicates that the absorption at 260 nm is more affected by scattering than absorption at 280 nm.

The specific radioactivity of the Ad 2 and Ad 12 particles was determined for each label used. The specific infectivity of the Ad 2 and Ad 12 was determined by plaque assay on HEK cells. These values are similar to those reported by Lawrence and Ginsberg (1967) for Ad 5 but slightly higher than values reported by Green et al. (1967a), Philipson (1967), and Lonberg-Holm and Philipson (1969) for Ad 2; and similar to the values reported for Ad 12 by Green et al. (1967a).

The buoyant density and sedimentation coefficient of the Ad 2 and Ad 12 were determined in the analytical ultracentrifuge. The sedimentation coefficient was determined in 1 M NaCl in D_2O to avoid aggregation. The values listed in Table 13 are corrected to $S_{20,w}$. Green (1962) reported the $S_{20,w}$ of Ad 2 as 795 S. The buoyant density of these virus particles has been reported by Piña and Green (1965) to be 1.33 gm/cm^3 for Ad 12 and 1.34 gm/cm^3 for Ad 2.

The adenovirions were usually quantitated by the absorption at 260 nm. The correlations between this determination and the amount of infectivity, protein, DNA, and radioactivity when labeled with thymidine-^{3}H are listed in Table 14.

b. Quality of the adenovirus DNA. The data in Tables 10 and 11 indicate that the adenovirus DNA may contain 0.007% cell protein and 0.002% cell DNA. The experiment with L-methionine-^{35}S label indicates that the extracted Ad 2 DNA contained less than 0.02% total protein.

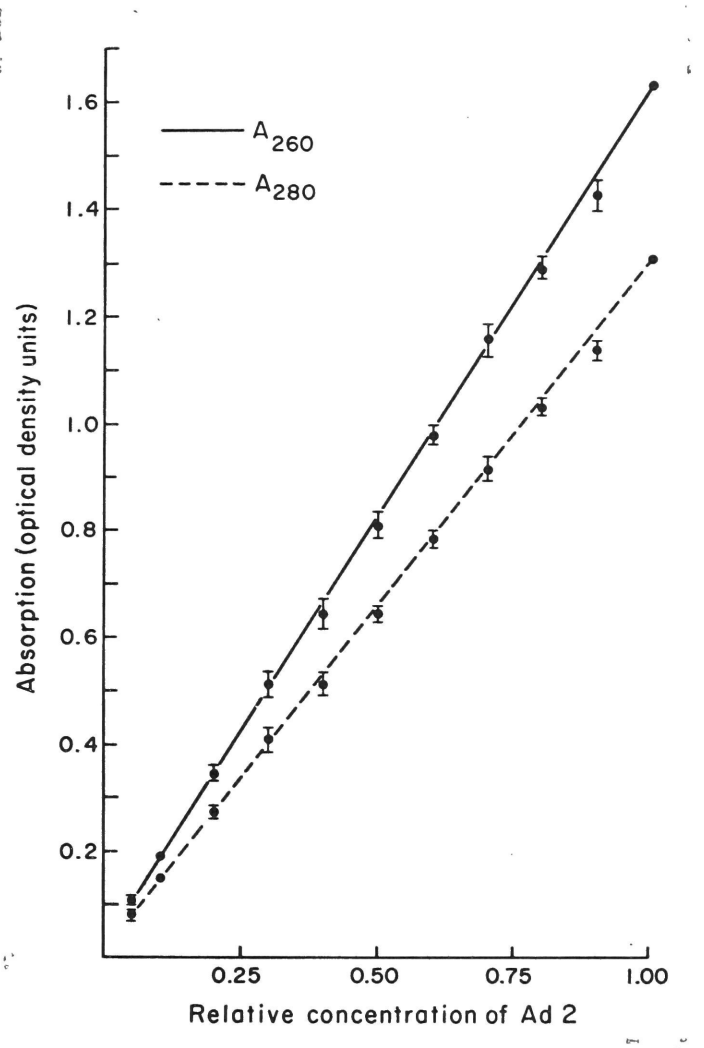

Figure 7: Linear relationship between absorption and concentration of adenovirus type 2. Absorption was determined in a quartz microcuvette with a 1 cm light path.

Table 14

Correlations between Physical Characteristics used to Quantitate the Adenovirions and Adenovirus DNA

	Ad 2	Ad 2 DNA	Ad 12	Ad 12 DNA
A_{260}	1.00	1.00	1.00	1.00
A_{280}	0.76 ± 0.12[e] N = 27	0.48 ± 0.09 N = 16	0.81 ± 0.17 N = 14	0.49 ± 0.12 N = 9
µg of protein				
chemical[a]	180	-	194	-
absorption[b]	207 ± 35 N = 27	-	205 ± 47 N = 14	-
µg of DNA[c]	26.4 N = 3	47.1 N = 3	21.8 N = 3	47.1 N = 3
Infectivity (PFU)	2.15×10^{10} N = 6	-	1.05×10^{10} N = 4	-
Radioactivity[d] (dpm)	$4.8 \times 10^5 \pm 4.1 \times 10^5$ N = 18	$6.5 \times 10^5 \pm 2.57 \times 10^5$ N = 21	$5.8 \times 10^5 \pm 3.6 \times 10^5$ N = 12	$7.6 \times 10^5 \pm 2.8 \times 10^5$ N = 15

[a] Determined by the method described by Lowry et al. (1951).

[b] Determined from the tables of Warburg and Christian (1941).

[c] Determined by the method described by Burton (1956).

[d] Uptake of thymidine-^{3}H.

[e] Mean ± SEM N = number of determinations

The physical parameters which define the Ad 2 and Ad 12 DNA are listed in Table 13. The ratio of absorption of the DNA at 260 to 280 nm is nearly 2.00 which is expected for pure DNA. The specific radioactivity of the DNA was determined for the isotopic labels used. The adenovirus DNA was not infectious for cells and no one has reported infectious DNA from human adenoviruses. The buoyant density and sedimentation coefficient for the adenovirus DNA was determined in the analytical ultracentrifuge. The buoyant density and sedimentation coefficient were reported by Green et al. (1967) for Ad 2 to be 1.716 g/cm^3 and 31.1 S for native and 34.1 S for denatured DNA, respectively. Adenovirus type 12 was reported to have a buoyant density of 1.708 gm/cm^3 and 30.6 S for native and 33.5 S for denatured DNA.

The viral DNA was usually quantitated by the absorption at 260 nm. The correlations between the absorption and concentration of DNA and radioactivity are listed in Table 14.

D. Preparations of Adenovirus Type 2 Capsid Subunits

Capsid subunits from Ad 2 were prepared and supplied by Drs. U. Pettersson and L. Philipson, Uppsala, Sweden. The penton, fiber and hexon subunits of Ad 2 were prepared by the methods described by Philipson (1960), by Pettersson et al. (1967, 1968), and by Pettersson and Höglund (1969). Briefly, KB cells infected with Ad 2 at a multiplicity of 10 to 25 PFU/cell were pelleted 72 hr after infection. The cells were resuspended in 0.01 M tris-HCl, pH 8.1. The cells were frozen and thawed once and further disrupted by expulsion through a 16 gauge needle. The cellular debris was removed, the cell homogenate was then extracted with trichlorotrifluoroethane. The intact virus was removed by zonal centrifugation in CsCl.

The soluble proteins which sedimented slowly were passed through a DEAE-cellulose chromatography column. Chromatography on DEAE separates the fibers, hexons and pentons into discrete fractions. Each subunit was further purified by gel filtration on 6% agarose and by preparative polyacrylamide gel electrophoresis. Each of the subunits was homogeneous by

analytical polyacrylamide electrophoresis at pH 8.0 and 9.5, boundary sedimentation in the analytical ultracentrifuge, and immunoelectrophoresis. When examined in the electron microscope, the subunits were morphologically intact and pure.

E. Preparation of Cellular DNA

Cells grown in suspension culture or cells which were suspended from monolayers by trypsin treatment, were pelleted and washed once with one volume of PBS. When labeled DNA was required, the cells were grown for 48 to 72 hr in the presence of thymidine-^{3}H or formate-^{14}C. The cells were resuspended at a concentration of 10^{6} cells/ml in 0.01 M tris-HCl at pH 8.1 and 0.005 M EDTA. The cells were lysed with SDS, digested with pronase solution and extracted with phenol. The phenol was removed by two extractions with diethyl ether. The DNA solution was placed in a nitrocellulose S40 centrifuge tube, about 1 g CsCl/ml solution was added and the mean density was adjusted to 1.70 g/cm^{3}. The mixture was centrifuged for about 1.0 million g-hr, the tube was punctured and fractions were collected from the bottom. The fractions which contained DNA were identified by the A_{260} or by the radioactivity of the DNA. The DNA was dialyzed against 1000 volumes of buffer.

The cellular DNA had an A_{260}/A_{280} ratio close to 2.00. The specific activity of the tritiated cellular DNA was usually about 5 nCi/micromole. Before the cellular DNA was used as a substrate for the endonuclease assay, the DNA was sedimented through a 5 to 20% sucrose gradient in the SW 25 rotor. A 1 ml fraction near the bottom of the tube was collected and dialyzed. The DNA from this fraction sedimented homogeneously in neutral and alkaline sucrose density gradients.

F. Preparation of Bacteriophage

Escherichia coli B and bacteriophage T4 were generously supplied by Dr. M. Schweiger. The E. coli culture was grown in M-9 media to a concentration of 1 to 2 x 10^{8} bacteria/ml (1.0 ODU_{600} = 1.5 x 10^{8} cells/ml). The composition of the M-9 medium is listed in Appendix A. The T4 phage was produced and purified and the T4 DNA was extracted by the method

described by Adams (1959). The sedimentation coefficient of the T4 DNA was 81 S in neutral sucrose gradients and was close to the value of 58 to 79 S reported by Hearst and Vinograd (1961). The culture was infected with 1 x 10^5 phage/ml, and 1 μCi/ml thymidine-^{3}H and 25 mg/l L-tryptophan was added.

Bacteriophage f1 was a gift from Drs. E. F. Rossomando and N. D. Zinder. Circular and linear DNA prepared from bacteriophage lambda was supplied by Dr. W. Doerfler. The covalently linked circles of lambda DNA were prepared by the method described by Gellert (1967). The linear lambda DNA sedimented at 34 S in neutral sucrose, the supercoiled circular DNA from lambda sedimented at 65 S in neutral and 145 S in alkaline sucrose gradients. These values agree closely with those published by Gellert (1967).

G. Enzymes

1. Pronase. Pronase was supplied by the Calbiochem Co., Los Angeles, Calif. The enzyme was isolated by fractional precipitation from cultures of Streptomyces griseus and had a specific activity of 45,000 proteolytic units/gm. Nomoto et al. (1960a,b) have established that this broad spectrum protease will rapidly hydrolyze virtually any protein to free amino acids. The viral proteins were digested with pronase and the double-stranded DNA was released. Pfau and McCrea (1962) have described a similar method for vaccinia virus DNA. The pronase solution which contained 1 mg/ml pronase, 2 mM β-mercaptoethanol and 50 mM EDTA, was incubated at 37°C for 30 min before storage. This incubation degrades any protein impurities which might be present. Upon prolonged incubation with pronase, purified viral DNA did not show any alteration in size as determined by velocity sedimentation in neutral or alkaline sucrose density gradients. Thus the preincubated pronase was free of exonuclease or endonuclease activities.

2. Trypsin. Crystalline trypsin was supplied by the Nutritional Biochemical Corp., Cleveland, Ohio. The trypsin was crystallized two times and supplied as a salt-free lyophilized powder.

3. Deoxyribonuclease. Pancreatic deoxyribonuclease I was supplied by the Worthington Biochemical Corp., Freehold, N. J. It was electrophoretically purified and supplied as a lyophilized powder. The deoxyribonuclease was free of ribonuclease activity. The deoxyribonuclease was used at 10 µg deoxyribonuclease/ml of buffer (5 mM $MgSO_4$ and 100 mM acetate at pH 5.0).

H. Production of Antiserum to Adenovirions

Antiserum was produced against Ad 2 and Ad 12. The adenovirus which had been purified by three cycles of equilibrium sedimentation in CsCl density gradients was used as the antigen. New Zealand white rabbits, weighing about 2 kg, were injected intravenously with the adenovirus in CsCl. A small amount of CsCl injected intravenously into the rabbits caused cardiac failure and death. To avoid this problem, the rabbits were digitalized intravenously with 0.03 mg of ouabain per kilogram 1 hr before the antigen was injected. Then approximately 250 µg of adenovirus in 0.5 ml of 4 M CsCl were injected intravenously. Ouabain (0.02 mg/kg) was also added to the antigen.

The adenovirus antigen was injected twice, 7 days apart. Thirty days after the last injection of antigen, the rabbits were bled and the sera were prepared. Table 15 illustrates the neutralization titers of the antiserum. The antiserum would specifically neutralize only the homologous adenovirus.

I. Radioisotopes Used

1. Thymidine-6-^{3}H. Thymidine-^{3}H was supplied by the Amersham/Searle Corp., Des Plaines, Ill. The tritium is nominally on carbon 6 of the pyrimidine ring. The company states that up to 20% of the tritium may be of the methyl group. It is further claimed that the tritium cannot be removed by boiling for several hours in acid or alkaline solutions. All the thymidine-^{3}H had a specific activity greater than 25 Ci per millimole. The purity of random lots of thymidine-6-^{3}H was assayed on Brinkman MN-polygram, CEL-300 PEI, thin layer chromatography plates as described in Randerath (1966). The nucleotides were located under a UV light. The spots were cut out and the radioactivity was determined. More than 99% of the tritium

Table 15

Neutralization Titer of Antiserum to Adenovirus

Antiserum to	Neutralization titer[a] virus assayed: Ad 2	Ad 12
Ad 2	320	20
Ad 12	< 10	160

[a] The neutralization titer is the reciprocal of the highest serum dilution which reduced the number of plaque forming units by 50%.

was found with the carrier thymidine. Upon storage at 4°C, 1% of the thymidine-6-^{3}H may decompose per month. The labeled thymidine was usually used within one month of delivery.

When the thymidine-^{3}H was added to KB cell cultures infected with Ad 2, less than 1% of the tritium was adsorbed. Seven percent of the thymidine found in the infected cells was acid soluble. More than 80% of the acid precipitable thymidine-^{3}H found in infected cells was present in viral DNA.

2. Formate-^{14}C. Sodium formate-^{14}C was supplied by the Amersham/Searle Corp. or by Schwartz Bioresearch, Orangeburg, N. Y. The specific activity was 52 to 60 mCi per millimole and 99% of the ^{14}C was present as formate. When KB cells infected with Ad 2 were grown in medium containing formate-^{14}C, less than 1% of the label was taken up into the cells. Nearly 50% of the acid precipitable ^{14}C in infected cells could be recovered as purified virus. The DNA extracted from the ^{14}C labeled adenovirus contained 50 to 60% of the label and presumably the rest of the ^{14}C label was located in the viral protein.

3. L-proline-^{3}H. L-proline-^{3}H was supplied by the Amersham/Searle Corp. The specific activity of the uniformly labeled L-proline was 341 mCi per millimole and 98% of the label was present as L-proline. Between 2 and 5% of the L-proline was taken up from the medium by the cells. About 36.5% of the tritium label associated with the purified virion was found in the viral DNA.

4. Orthophosphate-^{32}P. Carrier-free orthophosphate-^{32}P was supplied by Amersham/Searle or Schwartz Bioresearch. The phosphate-^{32}P was used immediately; usually the experiments were finished within 2 weeks of delivery of the isotope. The $H_3{}^{32}PO_4$ was supplied in 0.1 N HCl. The solution was neutralized with 0.01 N NaOH before the isotope was added to cell cultures.

5. Other isotopically labeled precursors. L-leucine-4-5-^{3}H with a specific activity of 11.2 Ci per millimole, cytidine-5-^{3}H with a specific activity of 16.4 Ci per millimole, uridine-5-^{3}H with a specific activity of 30.2 Ci

per millimole, and L-methionine-^{35}S with a specific activity of 240 mCi per millimole were all supplied by the Amersham/Searle Corp.

J. Chemicals

1. Cesium chloride. Cesium chloride was purchased as the optical grade powder from the Harshaw Chemical Co., Cleveland, Ohio. The company certified that the contamination of the CsCl by other metals was less than .001% and the absorption at 260 nm was less than 0.02. Most heavy metals were present at less than one part per million. The complete analysis of trace metals in the cesium chloride is listed in Appendix C. The density of cesium chloride solutions was determined by pycnometry or calculated from the refractive index determined in a Bausch and Lomb refractometer thermoregulated to 25°C. The density of the cesium chloride solution was calculated from the refractive index by the equation and coefficients established by Ifft et al. (1961).

$$\rho^{25.0} = 10.8601\ \eta^{25.0} - 13.4974$$

2. Tris (hydroxymethyl) aminomethane. Primary standard and buffer reagent grade tris (hydroxymethyl) aminomethane (tris) was purchased from Sigma Chemical Co., St. Louis, Mo. The company certified that this preparation of tris contained less than 1 part per million of heavy metal ions. Buffers of the desired pH were made by neutralizing the tris base with HCl.

3. Ethylenediamine-tetraacetic acid. The disodium salt of EDTA was purchased from the Sigma Chemical Co., St. Louis, Mo. The EDTA contained less than 5 parts per million in trace metals.

4. Trichlorotrifluoroethane. Trichlorotrifluoroethane (Genesolv-D) was purchased from Allied Chemical, Morristown, N. J. The electronic grade was used.

5. Phenol. Analytical reagent grade phenol was purchased. The phenol was vacuum distilled twice and stored frozen at -35°C in sealed bottles wrapped with dark paper. Before use, the phenol was melted and saturated with PBS or TE buffer.

6. Diethyl ether. Analytical reagent grade diethyl ether was purchased in one-fourth pound cans. When a can was opened, the unused ether was discarded. The ether was tested and always found free of peroxides.

7. Sucrose. Special enzyme-grade sucrose was purchased from the Mann Research Laboratories, Inc., New York, N. Y. The recrystallized sucrose was free of deoxyribonuclease and ribonuclease activity and contained less than 1 part per million of heavy metals.

8. Sodium dodecyl sulfate. Sodium dodecyl sulfate (SDS) was supplied by Amend Drug and Chemical Co., New York, N. Y. as the USP grade.

9. Polyvinylpyrrolidone. Polyvinylpyrrolidone was supplied by the Sigma Chemical Co., St. Louis, Mo. The average molecular weight of the preparation was 360,000.

10. Ficoll. Ficoll was supplied by Pharmacia Fine Chemicals, Inc., Piscataway, N. J. The average molecular weight of the Ficoll was 400,000.

11. Bovine serum albumin. Bovine albumin powder was purchased from Armour Pharmaceutical Co., Kankakee, Ill. This preparation is Cohn's (Cohn et al., 1940, 1944) fraction V from bovine plasma.

12. β-Propiolactone. β-Propiolactone was purchased from K and R Laboratories, Inc., Plainview, N. Y. The preparation was certified 99.9% pure.

13. Actinomycin D. Actinomycin D was purchased from Calbiochem Co., Los Angeles, Calif.

14. Ethidium bromide. Ethidium bromide was supplied by Boots Pure Drug Co., Ltd., Nottingham, England.

15. Spermine. Spermine was purchased from Calbiochem Co., Los Angeles, Calif.

16. Cycloheximide. Cycloheximide (actidione) was purchased from Calbiochem Co., Los Angeles, Calif.

17. Ribonucleotides, deoxyribonucleotides and deoxyribonucleosides. 5-Fluoro-2'-deoxyuridine was a gift of Dr. W. E. Scott of Hoffman-La Roche, Inc., Nutley, N. J. 5-Bromo-2'-deoxyuridine was purchased from Calbiochem

Co., Los Angeles, Calif. All other nucleosides and nucleotides were purchased from P-L Biochemicals, Milwaukee, Wis.

18. Polynucleotides. The alternating copolymer of deoxyadenylic acid and thymidylic acid (dAT) was purchased from The Biopolymers Laboratory of General Biochemicals, Chagrin Falls, Ohio. Double-stranded poly dG:dC, composed of one-strand polydeoxyguanylic acid and one-strand polydeoxycytidylic acid was a gift of Dr. R. D. Wells, the University of Wisconsin, Madison, Wis. Both of these polynucleotides had a sedimentation coefficient of about 4 S, and thus a molecular weight of 60 to 70,000 Daltons (Studier, 1965).

K. Liquid Scintillation Fluid

1. Bray's liquid scintillation fluid. Each liter of Bray's (1960) liquid scintillation fluid contained 60 g naphthalene, 4 g 2,5-diphenyloxazole (PPO), 0.2 g 1,4-bis [2-(5-phenyloxazolyl)] benzene (POPOP), 100 ml absolute methanol, 20 ml ethylene glycol and p-dioxane was added until the volume was 1.0 liter. The dioxane was spectral grade, the methanol and ethylene glycol were analytical reagent grade and the naphthalene, PPO, and POPOP were scintillation grade. The scintillation fluid was made 24 hr before use. Aqueous samples (0.1 to 1.0 ml) were prepared in 10 ml of Bray's scintillation fluid. The spectral characteristics of the samples prepared in Bray's scintillation fluid are summarized in Appendix B.

2. Toluene-Liquifluor$^{(R)}$ scintillation fluid. Forty-two milliliters of Liquifluor$^{(R)}$, supplied by Pilot Chemicals, Inc., Boston, Mass., was added to each liter of toluene. Each liter of toluene based scintillation fluid contained 4 g PPO and 50 mg POPOP. The toluene based scintillation fluid was used for dried precipitated samples.

L. Preparation of Dialysis Tubing

Dialysis tubing 6.0 mm in diameter was purchased from the Union Carbide Corp., Food Product Division, Chicago, Ill. All dialysis tubing was rinsed in distilled water and stored in 0.005 M EDTA. Before use, the tubing was boiled in 10% sodium carbonate for 10 min, thoroughly rinsed with distilled water and then boiled for 10 min in distilled water. The UV adsorption and pH of control solutions placed in the dialysis tubing remained unaltered.

III. METHODS

III. Methods

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III. METHODS

A. Biological Methods

1. Induction of tumors in hamsters. Tumors were induced in hamsters by the method described by Larson et al. (1965) modified for use with Ad 2 and Ad 12. The virus preparations were purified by CsCl equilibrium sedimentation gradients at least three times. Each preparation contained a single species of particles when analyzed in the analytical ultracentrifuge by equilibrium sedimentation. The virus preparation was dialyzed against PBS for 8 hr immediately before injection into the hamsters. The concentration of the adenovirus preparation was determined by the absorption at 260 nm.

Pregnant hamsters were housed individually. The newborn hamsters, less than 24 hr of age were injected with 0.05 ml of virus subcutaneously in the nuchal region. The hamsters were examined frequently for tumors. The hamsters which died after the first week were autopsied and animals with large tumors were sacrificed. Tissues for histopathology were fixed in 10% formalin and the paraffin sections were stained with hematoxylin-eosin.

2. Survey for contamination by mycoplasma and bacteria. Cell cultures were surveyed weekly for contamination by Mycoplasma. A 1 ml sample of cells and culture medium was spread on a petri plate which contained 2.5 ml horse serum, .25 ml penicillin (10,000 units per ml) and 15 ml Pfannstil agar at pH 8.0. The preparation of Pfannstil agar is described in Appendix A. The inoculated plate was incubated in an atmosphere of 5% CO_2 at 37°C and was examined daily for five days under the microscope at 120 x magnification for mycoplasma growth.

Surveillance for bacterial contamination was also done weekly. One milliliter each of cell suspension and culture medium was spread on a blood agar plate and added to a tube of thioglycolate broth. The plate and broth were incubated at 37°C for 2 to 3 days and were examined for growth of bacteria.

3. Plaque assay of adenovirus. The infectivity titer of adenovirus preparations was determined by a modification of the plaque assay described by Strohl et al. (1967) and Rouse et al. (1963). Primary HEK cells in 60 mm petri dishes were grown in Eagle's medium supplemented with 10% calf serum until confluent. The adenovirus preparation was diluted in PBS which contained 0.2% bovine serum albumin. The dilutions were prepared and kept in an ice bath until used. The HEK cell monolayers were washed with PBS once, and the plates were inoculated in duplicate with 0.5 ml of the diluted virus preparation. The adenovirus was adsorbed for 2 hr at 37°C and the plates were tilted every 15 min. After the adsorption period, the inoculum was removed and the monolayer was overlayed with 10 ml of HEK overlay (5.0 ml of melted 1.7% purified agar, 3.5 ml of HEK medium and 1.5 ml of fetal calf serum). The plates were incubated at 37°C for one week. The HEK cell monolayers were stained by adding 5 ml of melted HEK overlay which contained 0.1% neutral red. The plaques were marked and counted every 3 or 4 days during the next three weeks.

4. Infection of cell monolayers. Cell monolayers were infected by the technique described by Doerfler (1968), with slight modifications. Cells were grown in petri plates 60 mm in diameter to a confluent monolayer. The plates were washed three times with 5 ml of PBS at 37°C immediately before infection. Adenovirus, which had been purified by equilibrium sedimentation in CsCl, was dialyzed 4 to 5 hr against five changes of 1000 volumes of PBS. The specific radioactivity, absorption at 260 nm and the infectivity was determined from small samples. The virus in 1.0 ml of 0.2% bovine serum albumin in PBS was adsorbed at 37°C for 2.5 hr. The plates were tilted every 15 min during adsorption. At the end of the adsorption period, the inoculum was removed, the plates were washed eight times with 5 ml of PBS at 37°C and the pre-warmed maintenance medium was placed on the cells. No radioactivity or infectious virus was found in the fifth and successive washes.

After the incubation period, the total cellular DNA was harvested. The medium was removed and the monolayer was washed six times with 5 ml of PBS. No radioactivity was found in the third and successive washes. The

cells were lysed with 1 ml of 0.5% SDS in 0.05 M tris-HCl buffer at pH 7.5 and 0.01 M EDTA.

5. Extraction of DNA for analysis. Total intracellular DNA and DNA from assays which contained cell proteins were all prepared by pronase digestion. One volume of pronase solution was added to nine volumes of sample and the mixture was incubated for 30 min at 37°C. After digestion, the DNA was extracted three times with equal volumes of phenol and two times with five volumes of diethyl ether. When the minute amounts of phenol which remained could have interfered with the assay, the viral DNA was dialyzed against 0.01 M tris-HCl buffer at pH 8.1.

6. Chemical inhibition of cellular metabolism. The inhibition of macromolecular syntheses of BHK, HEL and KB cell lines by various dosages of three chemical inhibitors was determined by a preliminary experiment. The dosage which caused at least an 80% inhibition of cellular biosynthesis was used for the final experiment.

Synthesis of DNA was inhibited by thymidine. The medium was 0.1 to 100 μM thymidine. Two hours after this medium was added to cells growing in petri plates, 1 μCi cytidine-^{3}H per ml was added. Then 24 hr after the label was added, the cells were washed eight times with PBS and the final wash was checked for residual radioactivity. The cells were lysed with 1.0 ml of 0.5% SDS in 0.05 M tris-HCl buffer at pH 7.5 and 0.01 M EDTA and the lysed cells were extracted three times with phenol. The nucleic acids which had been extracted were digested in 0.3 M KOH for 2 hr, then one volume of 10% TCA was added and the suspension was filtered through a glass fiber filter. The filter was washed with 10 ml of 5% TCA and 10 ml of methanol. The radioactivity on the filter was determined with toluene based scintillation fluid.

Protein synthesis was inhibited by actidione. The culture medium contained 1.0 to 100 μg/ml actidione. Two hr after this medium was added to cells growing in petri plates, 1 μCi leucine-4,5-^{3}H per ml was added. Then, 24 hr after the label was added, the cells were washed eight times with PBS, lysed with 0.5% SDS, diluted with an equal volume of 10% TCA,

and the tritium in the precipitate was determined on glass fiber filters, as described above.

Synthesis of RNA was inhibited by 0.1 to 10 µg of Actinomycin D per ml. Inhibition was determined in a similar way from the uptake of uridine-^{3}H which was added to the medium at 1 µCi/ml. The effect of these inhibitors on cell biosynthesis is shown in Figure 8. In the experiment, thymidine was used at 5 mM, actinomycin D at 5 µg/ml and actidione at 2.5 µg/ml.

7. Preparation of the endonuclease from cells infected with adenovirus. Monolayers of cells grown in 60 mm diameter petri dishes were infected with either purified Ad 2 or Ad 12 as described above. At the time of harvest, the cells were washed three times with PBS, then 1 ml of PBS was added to the plate and the cells were frozen and thawed three times. A nitrocellulose centrifuge tube was sterilized by UV light, the suspended cell debris was placed in the tube and was overlayed with sterile mineral oil. After the sample was centrifuged at 100,000 g-hr, the supernatant fluid was collected through the mineral oil, the mineral oil was discarded and the sedimented material was resuspended in 1 ml of PBS. The resuspended pellet and the supernatant fluid were then assayed for endonuclease activity.

8. Assay of the endonuclease associated with adenovirions and extracts of cells infected with adenovirus. Virions and cell extracts were dissolved or suspended in PBS and assayed for endonuclease activity. The sample was mixed with 0.5 to 1.0 µg of labeled DNA substrate (about 50,000 cpm) in a final volume of 1.0 ml. Usually, Ad 2 or Ad 12 DNA was used as substrate. After incubation at 37°C, the reaction was stopped with one volume of phenol. Then the reaction mixture was extracted three times with phenol and two times with diethyl ether. The size of the DNA was determined, often both in neutral and alkaline sucrose density gradients (see Section III. B.1.).

9. Assay of the endonuclease associated with penton subunits. In preliminary experiments, viral subunits were assayed in PBS for endonuclease activity. In later experiments, the buffer system was better defined.

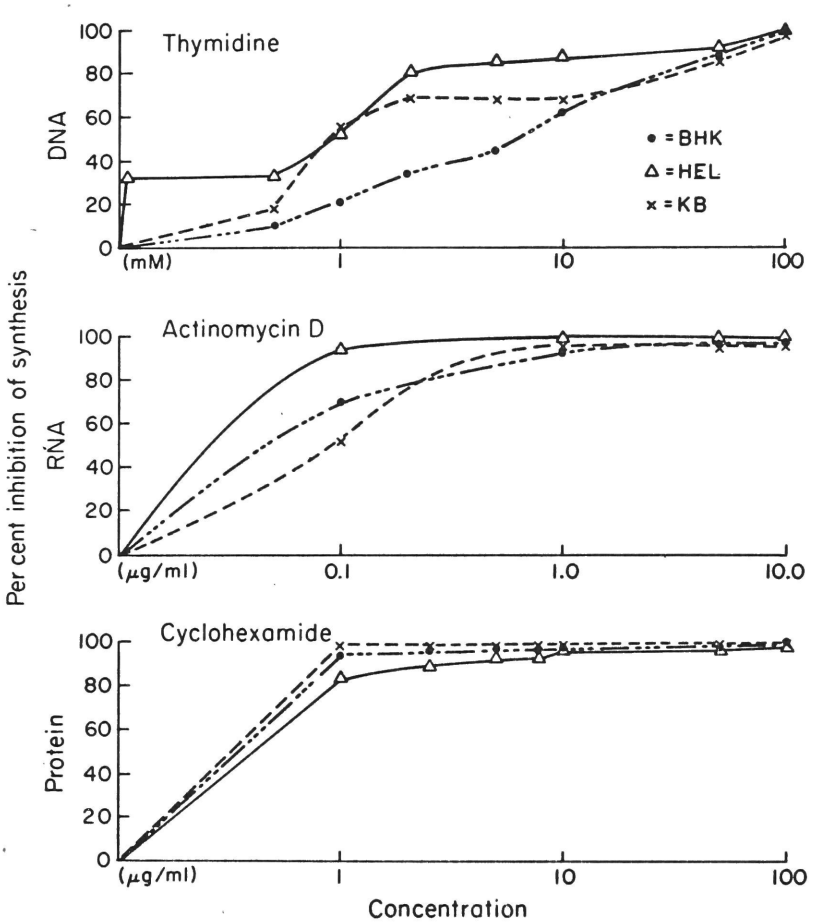

Figure 8: Chemical inhibition of cell metabolism. The KB, BHK and HEL cells in monolayer were treated with various doses of actinomycin D, actidione, and thymidine. The cells were treated with the inhibitor for 2 hr before the labeled substrate was added. The substrate and inhibitor were present in the medium for 24 hr and the amount of inhibition was determined for this period.

The standard reaction mixture contained in 1 ml; 1 μg of subunit, usually penton, 1 μg of Ad 2 DNA-^{3}H (about 20,000 cpm) in 10 mM tris-HCl buffer at pH 7.2, 100 mM NaCl, and 2 mM $MgCl_2$. The reaction mixture was prepared in an icebath and then incubated at 37°C for 30 or 120 min. The reaction mixture was sampled at intervals by removing aliquots of the reaction mixture and adding them to 0.5 ml 10 mM EDTA and 1 ml phenol in an icebath. This sample was then extracted three times with phenol and two times with diethyl ether. The size of the DNA was determined in neutral and alkaline sucrose density gradients (see Section III. B.1.).

B. Analytical and Preparative Methods

1. Zonal sedimentation in sucrose density gradients. The size of the viral or cellular DNA was determined by a modification of the zonal sedimentation method described by Burgi and Hershey (1963). Linear 5 to 20% (w/v) sucrose density gradients were used. The neutral sucrose solutions were made in 1.0 M NaCl, 0.01 M tris-HCl buffer at pH 7.2 and 0.001 M EDTA. The alkaline sucrose gradients were made in 0.3 M NaOH, 0.7 M NaCl and 0.001 M EDTA. The pH of the alkaline 5% sucrose solution was 12.8 and the pH of the alkaline 20% sucrose solution was 12.2.

The linear sucrose density gradients of 4.5 ml were formed in a Beckman SW 50 nitrocellulose centrifuge tube by a gradient maker modeled after the one described by Britton and Roberts (1960). For 3.5 ml gradients the Beckman SW 56 polyallomer centrifuge tubes were used. Thus nitrocellulose tubes were coated with Siliclad (Clay-Adams, Inc., New York). The DNA striking the walls was firmly attached to the wall and prevented from rapidly rolling to the bottom. Similar to the findings of Burgi and Hershey (1963), the silicone solution was effective when less than 1 μg of DNA was placed on the gradient. The polyallomer tubes could not be treated with silicone. Under optimal conditions, less than 0.5% of the DNA placed on the gradient was pelleted on the bottom due to interaction with the tube wall.

Marker DNA, usually 1 to 10 ng of Ad 2 DNA-^{14}C, was added to the 0.5 ml DNA samples, and the samples were carefully floated onto the top of the

sucrose gradient. The gradients were then centrifuged at 400,000 to 600,000 g-hr at 4°C, usually in a Beckman SW 50.1 or SW 56 rotor. After centrifugation, 9 drop fractions, about 0.20 ml each, were collected from the SW 50 tubes and 7 drop fractions, about 0.16 ml, were collected from the SW 56 tubes. All fractions were collected directly into scintillation vials from the bottom of the tube.

Extensive data establishing: 1) the linearity of the sucrose gradient, 2) the absence of deoxyribonuclease activity from the sucrose solution, 3) the conditions for isokinetic sedimentation, and 4) the accuracy of fraction collection, are described in Appendix D.2.

2. Determination of radioactivity and data processing. Ten milliliters of Bray's (1960) fluor was added directly to 0.15 to 0.20 ml samples from sucrose gradients and counted directly. Acid precipitated or desiccated samples were counted in toluene-Liquifluor(R) scintillation fluid.

The amount of radioactivity was determined in a Packard tricarb liquid scintillation spectrometer, model 3375. The amplification and window settings for optimal counting efficiency were determined for each fluor, isotope and type of sample. When two or three isotopes were used simultaneously, the settings which optimized separation of the isotopes but maintained high efficiency were used. A complete evaluation of the counting techniques is described in Appendix B.

Data from experiments which used two or three isotopes were processed by the Control Data Corporation 160 G digital computer. The exact methods and programs are discussed in Appendix D. Briefly, the data was automatically punched onto data cards by the scintillation spectrometer. The cards were scanned for errors by the computer, the errors were corrected and the corrected card images were transferred to magnetic tape for storage. The data were retrieved from the magnetic tape and processed by the discriminator ratio method derived by Okita et al. (1957).

The effect of the sucrose gradient on the counting efficiency and the spectrum of each isotope is discussed in Appendix B. The data were corrected for the effect of the gradient and the corrected data were printed

and plotted by the computer. A third-order interpolated fit of the data points was constructed and plotted. The maxima of the curve were found and the sedimentation coefficient for each maximum was calculated by the method of Martin and Ames (1961) and Burgi and Hershey (1963) using the marker DNA as a reference. The area under the curve of each maximum was calculated. The molecular weight of the native and alkaline-denatured DNA was calculated by the method of Studier (1965). The molecular weight of the virus was calculated by the method of Schachman and Williams (1959).

3. Equilibrium sedimentation in the analytical ultracentrifuge. Analytical equilibrium sedimentation was done by the techniques described by Vinograd and Hearst (1962). The CsCl gradients were analyzed in a Beckman model E ultracentrifuge, usually at 44,000 rpm and at 25°C. The sample was prepared in a CsCl solution in 0.1 M tris-HCl buffer at pH 7.2. When DNA was analyzed, 0.001 M EDTA was added. The refractive index of the sample and CsCl solution was determined with a Bausch and Lomb refractometer equilibrated at 25°C. Two degree Kel F centerpieces were used. Alternating deoxyadenylate-thymidylate copolymer was used as a reference for DNA samples and phage f1 was used as a reference for virus particles. The "absolute density" of phage f1 was determined as 1.3163 gm/cm^3. The details of this determination are presented in Appendix C.

When the sample was at equilibrium, photographs were taken on Kodak commercial film. The UV light source with the bromine-chlorine filter or the monochrometer at 265 nm was used. The photographs were traced with a Joyce-Loebl microdensitometer and the distance between the point of maximum concentration and the rotor reference mark was measured. The buoyant density was determined by the methods and equations of Ifft et al. (1961). The physical density gradient was corrected for the activity of the CsCl, the activity of the water and the specific angular velocity. The details of this calculation and the computer programs used for this calculation are explained in Appendix C.

4. Band velocity sedimentation in the analytical ultracentrifuge. Sedimentation coefficients were determined in a Beckman model E ultracentrifuge equipped with a high intensity, ultraviolet light source, ultraviolet optics

and an alternator attachment which allowed automatic photography of two cells during each experiment. The analytical method described by Vinograd et al. (1963) was employed.

Ultracentrifuge cells with 4 degree, 30 mm Kel-F centerpieces were used. Sample wells which could contain 0.04 ml were drilled into the top of each centerpiece as described by Studier (1965). When the cell was assembled, a fresh Kel-F gasket was placed on the top of the centerpiece. The sample which contained 1 to 2 μg of DNA was placed in the sample well, the top window was put into position, the cell was tightened, and 1.7 ml of bulk solution was placed in the cell. When the sedimentation coefficient of native DNA was determined, the bulk solution was 1 M NaCl, 0.01 M tris-HCl at pH 7.2 and .001 M EDTA. The sedimentation coefficient of denatured DNA was determined using 0.1 M NaOH, 0.9 M NaCl and .001 M EDTA as the bulk solution.

Studier (1965) states that the temperature of the aqueous samples in Kel-F centerpieces and the initial rotor temperature is equilibrated within 5 to 7 minutes. The temperature during sedimentation was determined by the rotor temperature indicator control, and these values were compared with the current temperature calibration curve to determine the temperature of the sample. There was a slow linear increase in rotor temperature between 21° and 22°C during the sedimentation experiment.

The samples were emptied from the wells by slow acceleration to 10,000 rpm. After rapid acceleration to 36,000 rpm, pictures were taken automatically with 5 sec exposures at 8 min intervals. The first photograph was usually taken about 10 min after the DNA lamella had formed. The wave length of the monochromater was set at 265 nm. Pictures were taken on Kodak commercial film, and the photographs were scanned with a Joyce-Loebl micro-densitometer. Since the distribution of the UV-absorbing material was symmetrically shaped with a clear maximum, the second moment of the DNA mass was assumed to be identical with the point of maximum absorption. All distances were measured from the reference mark to the point of maximum absorption.

The observed sedimentation coefficient was calculated from the equation:

$$\underline{S}_{obs} = 1/\omega^2 \; d \; (\frac{\ln \bar{x}}{dt})$$

$\underline{S}_{obs}$ = observed S value

ω = angular velocity in radians/sec

$\bar{x}$ = distance from the axis of rotation to the peak

All reported sedimentation coefficients are corrected to $S^\circ_{20,w}$ by the equation (Svedberg and Pedersen, 1940):

$$\underline{S}^\circ_{20,w} = \underline{S}_{obs} \cdot \frac{(\eta_{H_2O,\, t})}{(\eta_{H_2O,\, 20})} \cdot \frac{(\eta_{solvent})}{(\eta_{H_2O})} \cdot \frac{(1-\bar{V}\rho)_{20,w}}{(1-\bar{V}\rho)_t}$$

$\bar{V}$ = partial specific volume. 0.556 for the sodium salt of DNA (Hearst, 1962).

$\eta_{solvent}$ = viscosity of the solvent, values taken from Studier (1965) or determined by viscometry.

ρ = mean density of the bulk solution.

The $\underline{S}^\circ_{20,w}$ value was converted with the equations established by Studier (1965) to the molecular weight (M) of the native DNA ($\underline{S}^\circ_{20,w} = 0.0882\ M^{0.346}$) and denatured DNA ($\underline{S}^\circ_{20,w} = 0.0528\ M^{0.400}$).

The detailed methods and computer program used to calculate the $\underline{S}^\circ_{20,w}$ and the molecular weights are presented in Appendix D.

5. Equilibrium sedimentation of DNA in the presence of ethidium bromide. Equilibrium sedimentation in the presence of ethidium bromide was done by a modification of the method described by Radloff et al. (1967) and Bauer and Vinograd (1968). The sample was prepared in a 5 ml volumetric flask. The flask contained 3.66 g CsCl, 1 ng marker-^{14}C DNA (about 10,000 cpm), about 10 ng adenovirus DNA-^{3}H (about 10,000 cpm), 0.25 ml of ethidium bromide solution (10 mg/ml in 0.01 M tris-HCl at pH 7.2, and .001 M EDTA)

which made the final concentration of ethidium bromide 300 to 500 μg/ml and the volume was adjusted to 5 ml with buffer which contained 0.01 M tris-HCl at pH 7.2 and 0.001 M EDTA. The refractive index was adjusted to η_{25} = 1.3866 to 1.3870. Then the sample was transferred to a nitrocellulose centrifuge tube and overlayed with light mineral oil to 1/8 inch of the top of the tube.

The samples were centrifuged in the SW 50.1 rotor at 45,000 rpm for 48 hr at 15°C. Then the tubes were punctured, 5 drop fractions were collected, 10 ml of Bray's liquid scintillation fluid was added and the radioactivity was determined. Two drop samples were collected regularly while the gradient was fractionated. The CsCl gradient was determined from the refractive indices of these samples. The data were calculated and plotted using the "chromatography data" program described in Appendix D. The validity of the method was demonstrated with circular lambda DNA.

6. Zonal sedimentation of adenovirions in cesium chloride gradients. KB cells which had been infected with adenovirus were pelleted by centrifugation and washed with PBS. The cells were resuspended in 1 ml of PBS, and homogenized for 3 min with the microprobe of the Branson Sonifier at a setting of 2.5 amp. A discontinuous CsCl gradient was prepared by placing 1.0 ml of CsCl solution with a density of 1.50 g/cm^3 into a Beckman SW 50 nitrocellulose tube and carefully layering 3.0 ml of a CsCl solution with a density of 1.20 g/cm^3 over it. These CsCl solutions were prepared in PBS and the density was calculated from the refractive index. The cell extract, about 1 ml, was layered onto the top of the CsCl gradient. The gradients were centrifuged for 2 hr at 50,000 rpm in the SW 50.1 rotor. Any particle with a density greater than 1.28 g/cm^3 and a sedimentation coefficient greater than 75 S was sedimented onto the cushion of CsCl with a density of 1.50 g/cm^3. After centrifugation, several bands of particles could be seen at the interface of the two CsCl solutions. The particles at this interface were collected from the bottom of the tube.

7. DNA-DNA hybridization. The DNA-DNA hybridization procedure described by Denhardt (1966) was slightly modified and combined with the washing procedure described by Packman and Sly (1968). Schleicher and Schuell type

B-6 filters were heated to 80°C in 6 X SSC (SSC = 0.15 M NaCl and 0.015 M sodium citrate at pH 7.0) for 15 min to insure wetting. The filters were washed with 5 ml of 6 X SSC.

The DNA in 6 X SSC was denatured by heating for 10 min in a boiling water bath and quickly cooled by dilution into 100 volumes of ice cold 6 X SSC. The dilute, denatured DNA solution was passed through the filter at a rate of 5 ml/min and about 3 μg of the denatured DNA was adsorbed to the filter. Adsorption was more than 90% complete. The filter was washed with 10 ml of 6 X SSC, dried overnight in a vacuum desiccator and then placed in an oven at 80°C for 2 hr. The DNA was fixed onto the filter and less than 1% of the DNA eluted from the filter during the remaining procedures. The filter was preincubated in glass scintillation vials for 6 hr at 65°C in 1 ml of PM. (PM is 0.02% Ficoll, 0.02% polyvinylpyrrolidone, and 0.02% bovine serum albumin in 3 X SSC.) After incubation with PM, filters without DNA would adsorb less than 0.05% of the DNA in the incubation solution.

The labeled DNA in 6 X SSC to be hybridized to the DNA on the filter was sheared to about 10 S fragments with the Branson Sonifier with the microprobe with 6 amp output. The fragmented labeled DNA was denatured by heating, then quickly frozen in a dry ice bath, and about 0.25 ng (1000 cpm) of this denatured labeled DNA was added to the vials which contained the filters with DNA attached. After 12 hr of incubation at 65°C, the filters were washed six times with 20 ml of 0.01 M tris-HCl buffer at pH 8.1. Using this washing procedure, the nonspecifically adsorbed label was less than 0.1% of the input radioactivity. The amount of input DNA was determined by drying an aliquot of DNA on a filter. All samples were dried, and the amount of radioactivity determined in toluene-Liquifluor(R) scintillation fluid, and the percent of input radioactivity hybridized was calculated.

8. Protein and DNA determination. Protein concentrations were determined chemically with the method described by Lowry et al. (1951). Crystalline bovine serum albumin was used as a standard. DNA concentrations were

determined chemically with the method described by Burton (1956). Calf thymus or salmon sperm DNA was used as a standard.

The protein and DNA concentrations of small or dilute samples were estimated from the absorption at 260 and 280 nm. The protein and nucleic acid concentrations were then calculated from the data of Warburg and Christian (1941). The concentration of Ad 2 DNA was determined chemically and was correlated with optical density at 260 nm. The mean of three determinations was 1.0 ODU_{260} = 47.1 µg DNA/ml. The DNA concentrations of small and dilute samples was calculated from this value.

IV. RESULTS:

CHARACTERISTICS OF AN ENDONUCLEASE ASSOCIATED WITH ADENOVIRUS TYPES 2 AND 12

IV. Results: Characteristics of an Endonuclease Associated with Adenovirus Type 2 and 12

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IV. RESULTS:

CHARACTERISTICS OF AN ENDONUCLEASE ASSOCIATED WITH ADENOVIRUS TYPES 2 AND 12

A. Introduction:

Nucleases and Enzymes Associated with Viruses

Three groups of animal viruses, poxvirus, herpesvirus and reovirus, have virus coded enzymes which are associated with the virion during replication. All of these enzymes are related to the metabolism of nucleic acids.

1. Poxvirus. Most of these studies in the poxvirus group have been done with vaccinia virus, although two other poxviruses, rabbitpox and cowpox, have similar enzymes. Joklik (1964) and Dales (1965) demonstrated that uncoating of poxviruses was a process which required protein synthesis. This finding implied that uncoating was mediated by proteins coded for by the host cell genome and induced by incoming virus particles.

Further studies have demonstrated that this conclusion was, at least in part, incorrect. The intact poxvirus particles synthesized mRNA in vivo before uncoating occurred. Kates and McAuslan (1966) demonstrated that the mRNA required for the early virus-coded enzyme, thymidine kinase, was synthesized in vivo in the intact particle, but the mRNA for DNA polymerase, a late virus-coded enzyme, was not synthesized until the particle was uncoated. Kates and McAuslan (1967) further demonstrated that isolated vaccinia cores would synthesize early mRNA in vitro but not late mRNA. The authors concluded that the vaccinia core contained a specific DNA-dependent RNA polymerase. Basilico and Joklik (1968) have described a temperature sensitive mutant of vaccinia which produced only one-fourth as much early mRNA under restrictive conditions as under permissive conditions. This mutant should allow detailed studies on the role and origin of the RNA polymerase within the vaccinia core.

Five virus-coded enzymes have been identified in poxvirus infected cells: RNA polymerase (Kates and McAuslan, 1966, 1967), an acid deoxyribonuclease (McAuslan et al. 1965), an alkaline deoxyribonuclease (McAuslan,

1965), a neutral deoxyribonuclease (Jungwirth and Joklik, 1965), and a nucleotide phosphorylase (Gold and Dales, 1968). All five of these enzymes have been associated with purified vaccinia virions. The deoxyribonucleases were found outside the cores but within the envelope by Pogo and Dales (1969a). The authors suggest that these late viral enzymes were fortuitously enclosed within the envelope at the time of particle maturation and these enzymes do not represent structural components. However, Pogo and Dales (1969b) demonstrated that the RNA polymerase and the nucleotide phosphorylase were early enzymes and were enclosed within the core of vaccinia and may represent structural components.

2. Herpesvirus. The herpesviruses code for at least two deoxyribonucleases: an alkaline exonuclease described by Morrison and Keir (1968a) and an acid exonuclease described by Morrison and Keir (1968b), and Polin and Morrison (1969). The acid exonuclease is associated with but separable from the herpesvirus DNA-polymerase. The purified DNA-polymerase from herpesvirus also hydrolyzed DNA when at acid pH (Polin and Morrison, 1969).

3. Reovirus. Skehel and Joklik (1969) reported RNA-polymerase activity associated with the core of reovirus particles. The cores prepared from purified reovirions were able to synthesize all ten segments of the reovirus genome and there was at least one RNA-polymerase molecule per RNA segment. The reovirus RNA-polymerase has not been localized to a particular core protein.

The evidence presented in this section will characterize the size of the DNA molecules found in adenovirus infected cells and will lead to the description of a structural subunit of the adenovirion, the penton, which hydrolyzes double-stranded DNA.

B. Results

1. Metabolism of the parental adenovirus type 2 or adenovirus type 12 DNA in cells. The fate of the parental adenovirus DNA can easily be followed in vivo when cells are infected with adenovirions which contain labeled DNA. Adenovirus grown in the presence of thymidine-6-^{3}H was labeled only in the DNA. After the virus was purified by isopycnic,

equilibrium sedimentation in CsCl gradients, only the tritium label incorporated in the DNA remained. The CsCl was removed from the purified adenovirions by dialysis against PBS and cells were immediately infected with the virus. The virus was quantitated by determining: 1) the absorbancy of the solution at 260 nm, 2) the infectivity titer (PFU/ml), and 3) the amount of radioactivity per ml. The cells for infection were grown to confluent monolayers in 60 mm diameter petri plates. These cells were infected with the labeled virus and the total intracellular DNA was extracted at various times after infection. The DNA was extracted and its size was determined in alkaline and neutral sucrose density gradients (see Section III. B.1.). The number of cells on mock-infected plates was determined by suspending the cells with trypsin-versene and counting the cells in a hemocytometer.

Each plate contained between one and five million cells. The infectivity titer of the adenovirus was about 10^{10} to 10^{11} PFU/ml. Thus the multiplicity of infection was about 10^4 to 10^5 plaque forming units per cell or about 10^6 to 10^7 adenovirus particles per cell. However, only a small portion of the inoculum was adsorbed by the cells.

The virus inoculum for each plate usually contained about one million counts per minute but only about one to two thousand counts per minute remained associated with the cells after adsorption and exhaustive washing with PBS. Figure 9 shows the amount of label associated with the cells and found in the medium during an experiment in which BHK cells were infected with Ad 12-^{3}H. More than 98% of the label found in the medium was acid precipitable and furthermore, nearly half of the label is present in infectious particles. More than 90% of the label associated with the cells is acid precipitable. Similar results are found with HEK, KB, HEL and HeLa cells infected with either Ad 2 or Ad 12. Lawrence and Ginsberg (1967) reported that 15% of the Ad 5 associated with KB cells was eluted by 8 hr after infection. Thus a major portion of the adenovirions which is associated with the cells, is eluted from the cells in an infectious form during such in vivo experiments.

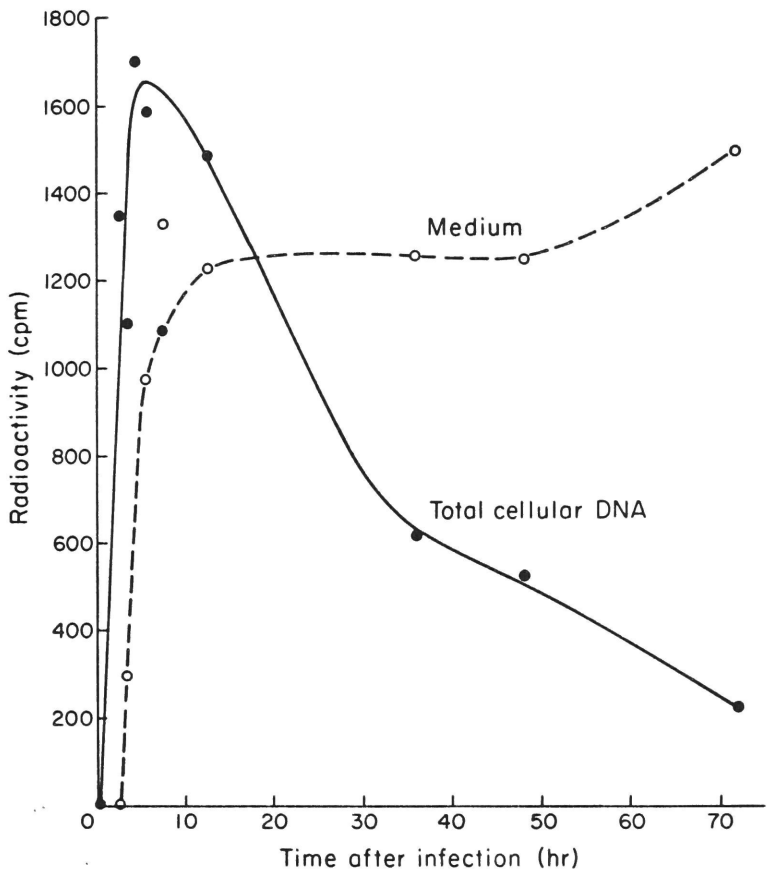

Figure 9: Distribution of tritium label in medium and in BHK cells infected with Ad 12 labeled with thymidine-6-^{3}H. Each plate contained 4.7 x 10^{6} cells and each plate was inoculated for 2-1/2 hr with an aliquot of Ad 12 which contained 5 x 10^{5} cpm. The plates were exhaustively washed and medium was added. After various intervals of incubation, the medium was removed, and the plate was washed five times. The cells were lysed with 1.0 ml 0.5% SDS, digested with pronase and the acid precipitable material counted.

The total intracellular DNA was extracted and the size of the labeled DNA from the parental virus was determined by zonal sedimentation. The sedimentation pattern of the label extracted from BHK cells 12 hr after infection with Ad 12-^{3}H is shown in Figure 10. Three species of labeled DNA are found both in alkaline and neutral gradients: A fast sedimentating component, here about 80 S, a labeled component cosedimenting with the marker DNA and a slower sedimenting component, here about 18 S. Each of the DNA species is symmetrically distributed and is apparently homogeneous. The three species of DNA found in the alkaline gradients have the sedimentation coefficients expected from their sedimentation behavior in neutral gradients. This type of pattern is found for each of the cell lines investigated, BHK, KB, HEK, HEL and HeLa cells, which are infected with either Ad 2 or Ad 12.

Presumably, the fast sedimenting DNA represents the integrated form of the adenovirus DNA described by Doerfler (1968). Since an equal number of counts of marker was added to the sample before sedimentation and little or no marker label was found in the fast sedimenting DNA, the fast sedimenting DNA is not the result of block sedimentation or sedimentation of DNA which collided with the tube wall and rolled to the bottom of the tube. This is supported by the fact that the tubes were either coated with Siliclad to which the DNA remains attached or the tube walls were not wettable and thus little interaction between the DNA and the tube wall occurred. The data from the alkaline gradients support the claim that the fast sedimenting DNA is covalently linked to large fragments of cellular DNA. The fact that the productively infected cells as well as the abortively infected cells contain fast sedimenting labeled DNA suggests that covalent linkage of the viral genome to the cellular genome may not be an unusual or special event which occurs only in transformation by the adenoviruses.

The labeled DNA which cosediments with the marker DNA extracted from purified adenovirus particles must represent the parental adenovirus DNA which remains unchanged in size. Since this DNA also cosediments with the marker DNA in alkaline gradients, it has few if any single-strand breaks.

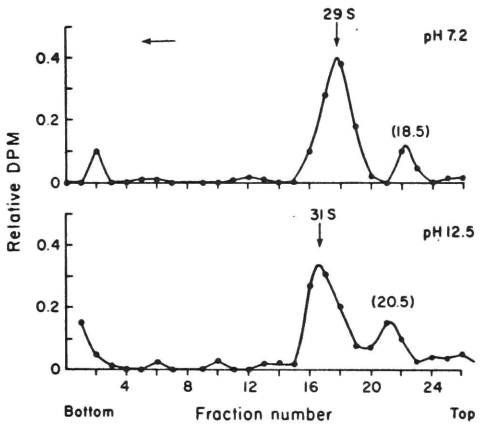

Figure 10: Sedimentation of labeled DNA extracted from BHK cells 12 hr after infection with Ad 12 labeled with thymidine-^{3}H. The multiplicity of infection was 4 x 10^{3} PFU/cell. The arrow indicates the position of the marker DNA in alkaline and neutral sucrose gradients.

This DNA could be derived from uncoated viral DNA in the cell, from intact virions which are adsorbed onto the surface of the cell, or from partly uncoated virions. The state of the virions in which this DNA is found will be described in greater detail later. In all the cell types investigated and at times from 2 to 24 hr after infection, this is the predominant species of parental DNA found.

The slow sedimenting DNA has a symmetrical profile when sedimented in sucrose density gradients at both pH 7.6 and pH 12.5. This species of DNA probably corresponds to the slow sedimenting Ad 12 DNA from BHK cells reported by Doerfler (1968a,b; 1969).

The adenovirus DNA may have been fragmented by the manipulations in the extraction procedure. Thus the size of the viral DNA was determined for four procedures. The DNA solution could flow by gravity through a Pasteur pipette up to 40 times without causing double- or single-strand breaks. However, forceful flow through a Pasteur pipette with a dropper bulb did cause single- and double-strand breaks in the DNA. After 20 forceful pipettings, one-third of the DNA molecules had new single-strand breaks. When the DNA solution was transferred through a micropipette (100 µl) 20 times, about 20% of the DNA had new double- and single-strand breaks. Sonication of the DNA at low power, 2.2 amp, for even 10 sec completely fragmented the DNA at random. This control established that the limited manipulations used in the experiments did not alter the size of the DNA.

2. Characteristics of the three species of adenovirus type 2 and adenovirus type 12 DNA found in infected cells.

a. Fast sedimenting species of adenovirus DNA. The sedimentation coefficient of the fast sedimenting species of Ad 2 or Ad 12 DNA varied between 45 S and 80 S. Apparently this variation was due to mechanical breakage and was related to the way the cellular DNA was manipulated. The fast sedimenting component is also found in alkaline sucrose gradients. The sedimentation coefficient for the denatured form of the DNA is that predicted from the sedimentation coefficient of the native form. Thus the cellular DNA has few additional single-strand breaks. The labeled DNA was

identified by DNA-DNA hybridization. The fast sedimenting DNA was isolated from alkaline sucrose density gradients, sheared and hybridized with cellular and viral DNA. For this experiment, HEK cells infected with Ad 2 were used since the uptake of adenovirus is the highest in these cells and enough labeled DNA for accurate hybridization assays can be obtained.

The results of the DNA-DNA hybridization are listed in Table 16. Three-tenths of one percent of the DNA from uninfected HEK cells hybridized to Ad 2 DNA but 22.0% of HEK cell DNA annealed to HEK cell DNA. About 50% of the control Ad 2 DNA hybridized with Ad 2 DNA. At 8 hr after infection, 6% of the fast sedimenting label adsorbed to Ad 2 DNA and 3.0% adsorbed to HEK cell DNA. However, at 24 hr after infection, 15% of the label annealed with Ad 2 DNA and less than 0.1% adsorbed to the HEK cell DNA. These data indicate that the fast sedimenting label constitutes viral genetic material. The hybridization of the fast sedimenting DNA to viral DNA is < 0.1 to 20% in HEK, BHK, and KB cells infected with Ad 2 or Ad 12. The hybridization to cellular DNA is variable and not very reproducible, and ranges from < 0.1 to 5% of the label adsorbing to the cellular DNA.

This low level of hybridization of the fast sedimenting DNA could be explained in three ways: 1) The viral DNA could be hydrolyzed and the resulting tritiated TMP reincorporated into cellular DNA. If this were so, an increased amount of hybridization to cellular DNA would be expected with time after infection. However, this is not found. 2) The viral DNA may be replicated as polymeric forms and reincorporate the label. However, the postulated polymeric forms of adenovirus DNA should hybridize to viral DNA and not to cellular DNA. 3) The random sonic fragments of cellular DNA which contain integrated viral DNA could contain both labeled viral DNA and unlabeled cellular DNA. Such fragments would hybridize to both viral and cellular DNA. The low and nonreproducible level of hybridization could be explained by the low concentration of the labeled form in a large amount of unlabeled cellular DNA. Thus the likelihood of a DNA fragment containing both cellular and viral DNA hybridizing

Table 16

Hybridization of DNA Isolated from HEK Cells

Infected with Adenovirus Type 2 Labeled with Thymidine-6-^{3}H

DNA-^{3}H hybridized	Percent of DNA-^{3}H from HEK cells adsorbed to filters			
	Ad 2 DNA fixed to filter		HEK cell DNA fixed to filter	
	8 hr p.i.	24 hr p.i.	8 hr p.i.	24 hr p.i.
> 45 S	6.0	15.0	3.0	< 0.1
34 S	64.0	37.0	< 0.1	< 0.1
20.5 S	44.0	67.0	< 0.1	< 0.1
HEK, cellular	0.2	0.4	-	22.0

with cellular DNA on the filter is markedly reduced. A shear fragment containing mainly or entirely viral DNA would hybridize better to viral DNA on the filter since the competition is reduced and the relative amount of homology is higher. This explanation seems the most likely and would agree with the physical data on the integration of Ad 12 DNA into BHK cell DNA presented by Doerfler (1968a,b). However, the existence of a newly synthesized polymeric form of viral DNA cannot be rigorously excluded.

After BHK cells were infected with Ad 12, which was labeled with thymidine-6-^{3}H, the cells were lysed at various times by freezing and thawing three times. One-half of each sample was incubated for 60 min at 37°C with 100 µg of pancreatic deoxyribonuclease. The DNA from the deoxyribonuclease treated sample and untreated control was extracted and analyzed by velocity sedimentation in neutral sucrose gradients. These results are summarized in Table 17.

When the total cellular DNA of infected cells was treated with deoxyribonuclease, all of the fast sedimenting label was digested slowly. The small amount of label left after treatment may have been due to the large amount of DNA present in this fraction and the digestion was not complete. Thus the fast sedimenting DNA is uncoated within the cell.

b. The species of adenovirus DNA which cosediments with the intact viral DNA. The DNA which cosediments with the marker DNA is symmetrically distributed in both alkaline and netural sucrose gradients and sediments at 33 S and 29 S, respectively. According to Studier's (1965) calibration, a native double-stranded DNA molecule sedimenting at 29 S at neutral pH has a sedimentation coefficient of 33 S after denaturation at alkaline pH. Thus it appears that this species of DNA is native double-stranded DNA with essentially no single-strand breaks. These values compare with the sedimentation coefficients of 33.5 S and 30.6 S, respectively, determined in the analytical ultracentrifuge by Piña and Green (1965).

Table 17

Sensitivity of Parental Adenovirus DNA to Pancreatic Deoxyribonuclease after Infecting Cells

DNA species:	Fast sedimenting		Cosedimenting		Slow sedimenting	
Samples	Control[a]	DNAse treated[b]	Control	DNAse treated	Control	DNAse treated[c]
Ad 12 DNA			94.9	0		
Ad 12 DNA and BHK cell extract			83.1	0		
Ad 12 virus			95	90.4	4	0
Ad 12 virus and BHK cell extract			69	62.5	12	0
BHK cell extract 3 hr p.i. Ad 12	2.1	0.2	56	10	12	0
BHK cell extract 5 hr p.i. Ad 12	8.8	0	73	11	18	0
BHK cell extract 7 hr p.i. Ad 12	11	0.4	55	0	34	0
BHK cell extract 24 hr p.i. Ad 12	15	0.7	34	0	20	0

[a] Relative amount of the species of DNA.

[b] A plate which contained 10.3×10^6 BHK cells was infected with a multiplicity of infection of 10^5 and 617,000 cpm Ad 12. The freeze-thaw extracts of infected cells were incubated at 37°C for 60 min with 100 µg of pancreatic deoxyribonuclease, 0.01 M $MgSO_4$, 0.1% bovine serum albumin in PBS deficient. The value listed is the percent of DNA which remains after treatment.

[c] In deoxyribonuclease treated samples, nearly all of the label sedimented at less than 3 S.

The data in Table 16 indicate that this type of DNA hybridizes only to viral DNA and not to cellular DNA. The decreased hybridization at 25 hr after infection may be due to competition from newly synthesized unlabeled Ad 2 DNA in the system. It is concluded from the size of the DNA and the results of the DNA-DNA hybridization experiment that the DN isolated from infected cells which cosediments with the marker DNA is intact, double-stranded Ad 2 or Ad 12 DNA.

Table 17 indicates that all of the extracted Ad 12 DNA is sensitive to digestion with deoxyribonuclease while only 4.6% of the DNA in the virus particle is sensitive to deoxyribonuclease. This compares with 3.2% found by Philipson (1967) for Ad 2, and 3% found by Lawrence and Ginsberg (1967) for Ad 5. When the virus is incubated at 37°C with the BHK cell extract, about 20% of the intact DNA is cleaved to slow sedimenting fragments and about 25% of the viral DNA becomes susceptible to deoxyribonuclease. Lawrence and Ginsberg (1967) found that 21% of Ad 5 with KB cell extracts becomes susceptible to deoxyribonuclease under similar conditions. The partial degradation of the virion during the in vitro incubation and exposure of the viral DNA may be partially explained by the action of proteolytic cellular enzymes.

Five hours after infection of BHK cells with Ad 12, essentially all of the DNA was susceptible to deoxyribonuclease. These data are similar to the uncoating data for Ad 5 in KB cells obtained by Lawrence and Ginsberg (1967). These data suggest that all of the Ad 12 particles found in the cells are uncoated. The synchrony of uncoating may not be high since 10% of the DNA remains resistant to deoxyribonuclease until 5 hr after infection. This DNA may represent adsorbed particles outside the cell or particles in lysosomes or vacuoles discussed by Morgan et al. (1969) and Chardonnet and Dales (1970a,b).

c. The slow sedimenting species of adenovirus DNA. The species of DNA which sediments more slowly than the marker DNA is symmetrically distributed in both neutral and alkaline sucrose gradients. The respective sedimentation coefficients are 18.5 S and 20.5 S. The sedimentation coefficient of denatured DNA expected for a double-stranded intact DNA

sedimenting at 18.5 S would be 20.5 S. Thus the slow sedimenting DNA species appears to be a double-stranded DNA molecules without any single-strand breaks. Using the Studier (1965) equations, the molecular weight of the intact native AD 2 DNA is calculated at 24.9 million Daltons and the denatured single-stranded DNA has a molecular weight of 11.9 million Daltons. The molecular weight of the 18.5 S native DNA fragment is calculated at 5.4 million Daltons and the molecular weight of the 20.5 S denatured DNA fragment is 2.8 million Daltons.

The results in Table 16 show that the slow sedimenting DNA hybridizes only to Ad 2 DNA and not to cellular DNA. The data in Table 17 demonstrate that all of the slow sedimenting DNA is susceptible to digestion with deoxyribonuclease anytime after its appearance. Thus the slow sedimenting DNA is native viral DNA without single-strand breaks which has a size about one-fourth the molecular weight of the intact adenovirus DNA.

Thus all three species of DNA are digested by deoxyribonuclease and thus are uncoated. All are double-stranded DNA with few single-strand breaks. The label in fast sedimenting DNA hybridizes to both cellular and viral DNA, but anneals with viral DNA to a much higher extent. The cosedimenting and slow sedimenting species hybridize only to viral DNA. Thus the labeled portion of these three species is derived from the parental DNA of Ad 2 or Ad 12.

3. Changes in the amounts of the three species of adenovirus DNA during infection. Although there is only one species of DNA in the adenovirions which infected the cells, at 12 hr after infection adenovirus DNA of three different size classes or species are found in the cells. This result is observed in four different cell lines infected with either Ad 2 or Ad 12. Monolayers of BHK cells were infected with Ad 12 labeled with thymidine-6-^{3}H. The total intracellular DNA was harvested at various times after infection and analyzed on neutral and alkaline sucrose gradients. The relative amounts of the three types of DNA at various times after infection can be determined from the relative areas under the curve. These data are illustrated in Figure 11.

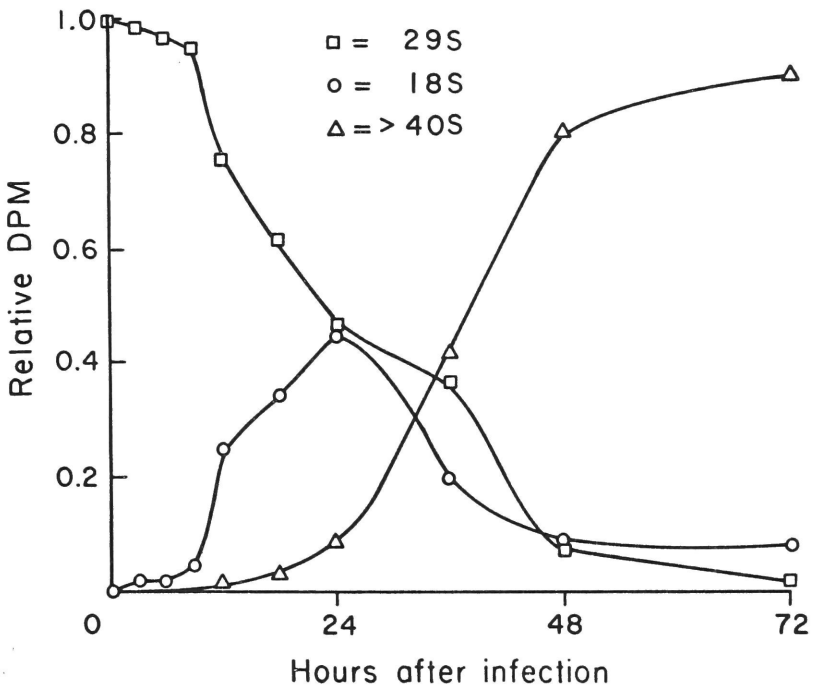

Figure 11: Relative amounts of each species of labeled Ad 12 DNA found in BHK cells after infection. Monolayers which contained 5.4×10^6 BHK cells were infected at a multiplicity of 3.6×10^3 PFU/cell with Ad 12 labeled with thymidine-6-^{3}H. At each time point, the total intracellular DNA from one monolayer was extracted and analyzed by zonal sedimentation on neutral sucrose density gradients.

During the first 12 hr after infection, only the intact Ad 12 DNA which sediments at 29 S is found. The 18 S DNA species first appears at 12 hr. As the amount of intact DNA declines, the 18 S species rapidly increases to a maximum amount at 24 hr after infection. At that time the 18 S DNA represents 45% of the labeled intracellular DNA. At 48 hr after infection, the amount of 18 S parental DNA declines to a constant value of about 8% of the labeled intracellular DNA. The fast sedimenting species of labeled DNA first appears in small amounts at 12 hr after infection. At 24 hr after infection, the amount of fast sedimenting DNA rapidly increases as the amount of 18 S DNA declines. At 72 hr after infection, 90% of the labeled Ad 12 DNA in the cell is found as the rapidly sedimenting form, 8% as the 18 S species and 1 to 2% as intact Ad 12 DNA. The same distribution was found in alkaline sucrose gradients. Similar results were found in BHK cells infected with Ad 2 and in HEL, KB, or HeLa cells infected with either Ad 2 or Ad 12.

Since the hybridization data demonstrate that the label in the three DNA species is in Ad 12 DNA, these data suggest that the parental Ad 2 or Ad 12 DNA is cleaved into 18 S fragments which may become linked to the fast sedimenting cellular DNA, presumably as the integrated form described by Doerfler (1968a,b). Thus the 18 S DNA may be the precursor of the integrated form of the Ad 12 DNA.

Since the results with cells infected by Ad 2 are similar, it is conceivable that the phenomenon of integration may also occur during productive infections, both with Ad 12 (a member of group A, highly oncogenic) and Ad 2 (a member of group C, nononcogenic). Thus, integration of the adenovirus DNA into host cell DNA may be a general event in either productive or abortive infections. The results further suggest that the formation of the fast sedimenting DNA and the 18 S DNA is a virus regulated phenomenon since the same species of DNA and the same relative amounts of each species are found in different cell types infected with Ad 2 or Ad 12.

However, as shown in Figure 9, the total intracellular Ad 12 DNA declines over the period of the experiment. Most of the label that is lost appears in the form of infectious virus particles which did not actually get into the cell. However, most of this loss occurs in the first 8 hr after infection. This loss of intact cell-associated virus may influence the slope of the curve for the decline of the 29 S DNA that actually is intracellular. There is no evidence for loss of the 18 S or the fast sedimenting DNA species into the medium.

4. Metabolism of newly synthesized DNA in cells infected with adenovirus type 2. The data in the previous sections demonstrate that the parental adenovirus DNA is distributed in three size classes. Then the size of the newly synthesized DNA in adenovirus infected cells was determined. Infected cells were pulse labeled with thymidine-^{3}H at various times after infection. Both BHK and KB cells were used for this experiment. Confluent monolayers of cells were infected with purified unlabeled Ad 2 at 116 and 160 PFU/cell, respectively. The controls were mock infected with PBS. The infected cells were incubated in maintenance medium. At intervals, monolayers were pulse labeled for 2 hr with 1 μCi of thymidine-^{3}H per ml. At the end of the pulse, the cells were washed and the total intracellular DNA was extracted and analyzed in neutral and alkaline sucrose density gradients. The distribution of newly synthesized DNA resembled that obtained for parental DNA shown in Figure 10. The cellular DNA in the mock-infected cultures was all found to be fast sedimenting.

The radioactivity was determined on a portion of each fraction from the alkaline sucrose gradients. The DNA in the alkaline sucrose gradient was divided into three classes: 1) fast sedimenting DNA (> 45 S), 2) intact Ad 2 DNA (33 S), and 3) slow sedimenting DNA (< 25 S). The relative amounts of these three classes of DNA as determined by zonal sedimentation in alkaline sucrose density gradients is seen in Figure 12. The fractions in each class were pooled, and the DNA in each fraction was hybridized both with cellular and with viral DNA.

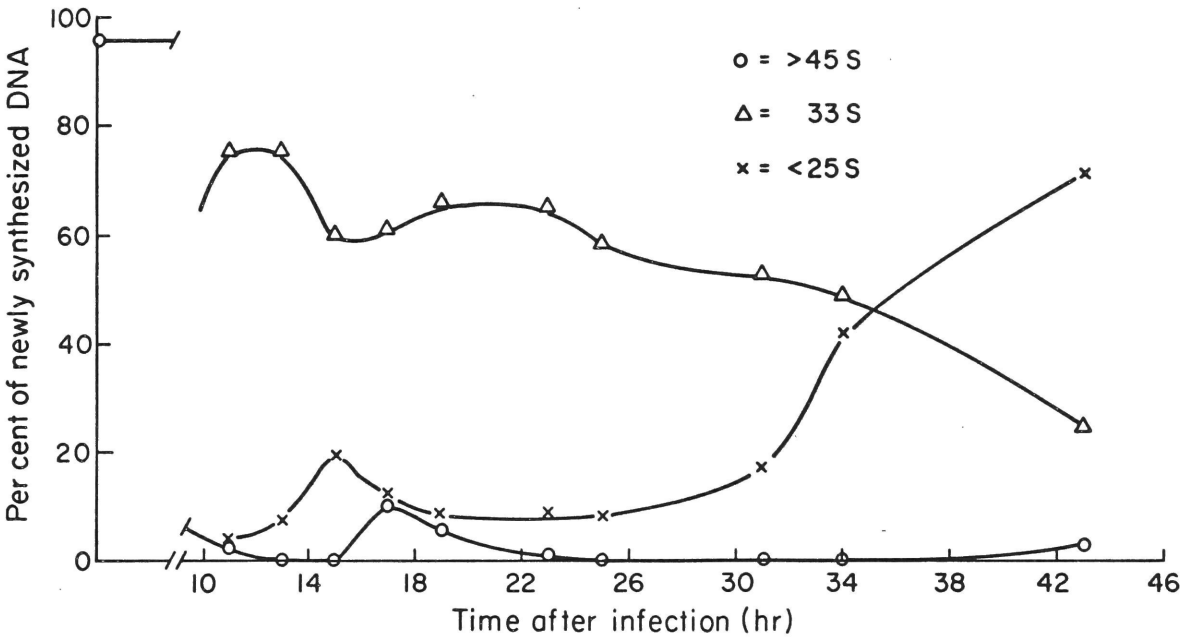

Figure 12: Relative amount of each species of newly synthesized DNA in KB cells infected with adenovirus type 2. Monolayers of KB cells were infected with 160 PFU of Ad 2 per cell. At intervals, plates were pulse labeled with thymidine-^{3}H for 2 hr. Then the total intracellular DNA was extracted and was analyzed on alkaline sucrose density gradients. The area under the curve for each of the three classes of DNA was calculated. The time points indicate the midpoint of the pulse labeling interval.

The pulse labeling was started at 10 to 12 hr after infection, a time when Ad 2 DNA should be rapidly synthesized. At that time the 33 S species of DNA was the predominant form, and constituted 50 to 75% of the newly synthesized DNA. The amount of 33 S species which was newly synthesized slowly declined until at 33 to 35 hr after infection, when it dropped markedly. The slow sedimenting DNA was synthesized and formed at 10 to 12 hr after infection and reached a maximum at 14 to 16 hr after infection and then declined. The slow sedimenting DNA became the predominant species late in infection, from about 33 to 35 hr after infection on.

Very little label was found in the fast sedimenting class of DNA although a small amount of newly synthesized fast sedimenting DNA at 16 to 18 hr after infection appeared. The fast sedimenting DNA which hybridizes with the Ad DNA could represent a small amount of polymeric form of Ad 2 DNA in the cell. Although there is no clear evidence to support this concept, there is a small amount (1 to 3%) of DNA in the alkaline and neutral sucrose gradients which sediments at about 45 S and thus represents molecules which contain 75 to 100 million Daltons of DNA. This DNA hybridizes only to viral DNA. The results of infecting BHK cells with Ad 2 were similar.

Again, early in infection the kinetic relationship of 33 S intact DNA to slow sedimenting DNA and slow sedimenting DNA to fast sedimenting DNA may suggest a precursor to product relationship. Later than 32 hr after the infection, the slow sedimenting form apparently represents a degraded form of the DNA, although it is all newly synthesized.

The data of Ginsberg (1964) and Green et al. (1964) indicate that host cell DNA synthesis is inhibited at the time viral DNA is synthesized. Thus all of the DNA synthesized after 10 hr should be viral DNA. DNA-DNA hybridization was done to identify the DNA in each class. The total amount of DNA synthesized during each 2 hr interval and the amount of labeled DNA in each size class annealing with cellular and viral DNA in a DNA-DNA hybridization experiment is seen in Table 18.

Table 18

Amount of Thymidine Incorporated into DNA and Identification of the DNA in the Three Classes of DNA from KB Cells Infected with Adenovirus Type 2

Hours after mock infection	cpm new DNA	Percent of DNA from alkaline sucrose gradient hybridizing with DNA on filter					
		Size of DNA in solution					
		> 45 S		33 S		< 25 S	
		DNA fixed on filter					
		KB	Ad 2	KB	Ad 2	KB	Ad 2
10-12	14,401	37	< 0.1	42	2.1	-	-
33-35	17,234	28	< 0.1	34	< 0.1	-	-
Hours after infection							
10-12	72,299	11	0.3	9.4	94	0.4	84
12-14	143,604	14	3.1	< 0.1	83	0.3	79
14-16	184,143	21	2.6	< 0.1	89	< 0.1	74
16-18	180,057	16	4.0	0.2	84	0.1	48
18-20	184,752	< 0.1	50	0.1	76	0.4	55
22-24	112,066	2.0	21	0.2	91	0.2	54
24-26	92,136	0.4	54	0.1	67	< 0.1	73
30-32	11,317	1.1	69	< 0.1	84	0.2	81
33-35	8,160	0.5	71	< 0.1	78	< 0.1	87
42-44	4,213	< 0.1	73	< 0.1	69	0.3	69

Control	DNA on filter		
DNA hybridized	KB	Ad 2	None
KB	67	0.2	< 0.1
Ad 2	0.3	84	< 0.1

The maximum amount of DNA synthesis occurs at 14 to 20 hr after infection. This finding is identical with that published by Green and Daesch (1961) and Mak (1969). The hybridization data show that nearly all of the DNA synthesized in all classes of infected cells is Ad 2 DNA. The DNA synthesized in mock infected cells does not hybridize with viral DNA. Little cellular DNA is synthesized when the Ad 2 DNA is synthesized. All of the newly synthesized cellular DNA appears in the fast sedimenting class. Only Ad 2 DNA is synthesized in the KB and BHK cells at times later than 16 to 18 hr after infection. The metabolism of the newly synthesized DNA is similar to the metabolism of the parental DNA in that three classes of DNA with comparable sizes are found. A precursor to product relationship is also suggested for the newly synthesized intact viral DNA being fragmented to slow sedimenting DNA and slow sedimenting DNA being integrated into fast sedimenting (cellular) DNA. The relatively small amount of newly-synthesized fast-sedimenting DNA is markedly different from the corresponding study following the parental Ad 12 DNA illustrated in Figure 11. This may be explained if the fast sedimenting label is integrated viral DNA and the integration process takes longer than the 2 hr of pulse. It is also striking that nearly all of the hybridization of the DNA in the fast sedimenting class after 18 to 20 hr after infection is with viral DNA. This may suggest synthesis of a polymeric form of adenovirus DNA.

5. Effect of chemical inhibitors of macromolecular syntheses on the metabolism of adenovirus type 12 DNA. The synthesis of DNA, RNA and protein in infected cells was inhibited to determine whether newly synthesized virus or cell products were involved in the formation of the fast or slow sedimenting species of viral DNA. The macromolecular syntheses of BHK cells in monolayers were inhibited by 5 mM thymidine, 5 μg/ml actinomycin D, or 2.5 μg/ml cycloheximide. At these concentrations the synthesis of DNA, RNA, or protein was inhibited 75 to 90% (see Section III. A.6.). The inhibitors were added to the BHK cells 2 hr before infection. The degree of inhibition was determined from the incorporation of tritiated cytidine, uridine or leucine in control cultures. The cells were infected with labeled Ad 12, the intracellular DNA was extracted at

12 hr after infection and analyzed by neutral sucrose density gradients. The amount of each species of DNA present was calculated. The results of this analysis are listed in Table 19.

The cells were treated with the inhibitors for 14 hr. Over this long period, the specificity of the chemical inhibitors was not very high except thymidine was a relatively specific inhibitor for DNA synthesis. Actinomycin D and cycloheximide caused a major reduction in DNA, RNA and protein synthesis. Inhibition of the cells with thymidine had little effect on the formation of the fast or slow sedimenting viral DNA. However, both cycloheximide and actinomycin D both inhibited the formation of the fast sedimenting DNA species but had little effect on the formation of the slow sedimenting species of viral DNA.

These results suggest that DNA, RNA and protein synthesis are not required for the formation of the 18 S species of viral DNA and that the endonuclease may be preformed within the cell or the adenovirion. However, since the inhibition of synthesis is not complete, the lack of inhibition of the endonuclease must be interpreted cautiously.

6. Effect of inactivation of virus on the metabolism of the parental viral DNA. Experiments were designed to elucidate whether the virus played an essential role in the mechanism which forms the slow sedimenting viral DNA in infected cells. Will the DNA of inactivated virus also be hydrolyzed? Adenovirus type 12 labeled with thymidine-^{3}H was inactivated by: 1) heat treatment, 2) ultraviolet light, 3) formaldehyde, or 4) β-propriolactone.

The labeled Ad 2 and Ad 12 were heat inactivated at 56°C for 30 min (Wilcox and Ginsberg, 1963a; Rafajko and Young, 1964). The metabolism of the DNA from heat inactivated virus was determined in BHK, HEL and KB cells. At 7 hr after infection, the size of the labeled DNA was determined. Typical results for BHK cells inoculated with heat inactivated Ad 12 are found in Figure 13. Similar findings are obtained with KB and HEL cells.

The data in Figure 13 show that nearly all of the labeled DNA found in the infected BHK cells sedimented at 16.6 S. Only a very small amount

Table 19

Amount of Parental DNA in Each Size Class
12 Hr After Chemically-Inhibited BHK Cells
Were Infected with Adenovirus Type 12

	Percent inhibition of synthesis:			Percent of parental DNA in size class:		
Inhibitor	DNA	RNA	Protein	> 40 S	29 S	18 S
None	0	0	0	12	67	15
Thymidine (5 mM)	75	15	4	9	70	19
Actinomycin D (5 µg/ml)	67	79	62	0	84	15
Cycloheximide (2.5 µg/ml)	62	76	90	0	83	16

The chemical inhibitor was added to the medium of monolayers of BHK cells 2 hr before infection. The monolayers were infected in the presence of inhibitor with 4.2×10^3 PFU/cell of Ad 12 labeled with thymidine-^{3}H. After adsorption for 2 hr, the cells were exhaustively washed and medium which contained the inhibitor was added. The monolayers were exhaustively washed again 12 hr after infection and the cells lysed. The DNA was extracted and analyzed by zonal sedimentation. The percentage of DNA in each size class was calculated. The degree of inhibition was determined from the uptake of leucine-^{3}H, uridine-^{3}H and cytidine-^{3}H.

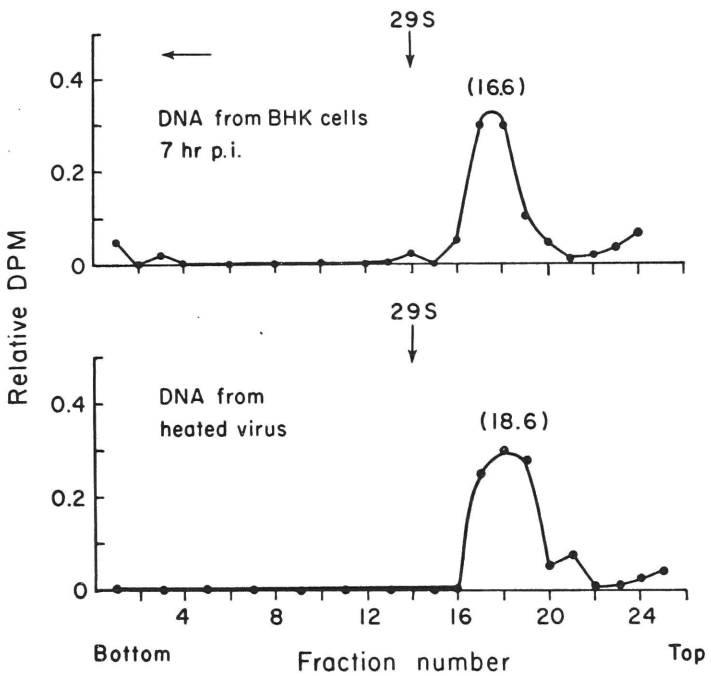

Figure 13: The metabolism in BHK cells of DNA from adenovirus type 12 which was inactivated by heat. Monolayers of 9.35×10^6 cells were inoculated with Ad 12 labeled with thymidine-^{3}H. This virus had an infectivity titer of 1.7×10^{10} PFU/ml, but after heat inactivation the infectivity titer was < 10 PFU/ml. The intracellular DNA was extracted 7 hr after infection and the size of the DNA was determined by zonal sedimentation on neutral sucrose gradients. DNA from an aliquot of the heat inactivated virus used as inoculum was also extracted and analyzed.

of the label sediments fast, cosediments with the marker DNA, or does not sediment at all. The DNA which sediments at 16.6 S is symmetrically distributed and appears to be relatively homogeneous.

When the DNA from the heated inactivated Ad 12 used as inoculum was analyzed, it sedimented at 18.6 S. No DNA sedimented faster than or cosedimented with the 29 S marker. These results clearly indicate that the adenovirus DNA is cleaved during the inactivation period and that the size of the viral DNA was only slightly reduced within the BHK cells. This further decrease in size (18.6 S to 16.6 S) may be due to an exonuclease found in BHK cells by Koh et al. (1970).

The data in Table 20 summarize the results of an experiment in which the uptake and elution of heat inactivated Ad 12 and the sedimentation coefficients of the labeled DNA were determined 7 hr after inoculation of BHK, HEL and KB cells. The uptake of the heat inactivated virus is increased 3- to 30-fold over the uptake of control virus. The amount of label which elutes from the cells inoculated with inactivated virus during the 7 hr incubation period is increased to about one-third to one-half of the adsorbed label. The enhanced uptake and elution of the heat inactivated virus may be explained by the formation of viral aggregates. Before heat inactivation, the viral suspension was clear, blue opalescent, but after heating it became markedly turbid and the color changes to a dull yellowish white.

Russell et al. (1967b) have shown that the DNA of adenovirions heated for 1 min to 56°C becomes accessible to pancreatic deoxyribonuclease. Thus the change in size of the Ad 12 DNA from 18.6 S to 16.6 S could have occurred in the cell without further uncoating of the viral DNA. The data of Morgan et al. (1969) and Chardonnet and Dales (1970a,b) suggested that virus particles which had been inactivated by physical or chemical means were sequestered in the lysosomes and were not uncoated normally. Lysosomal nucleases could be responsible for this further decrease in size of the viral DNA after infection.

Table 20

Uptake and Elution of Heat Inactivated Adenovirus Type 12 and Sedimentation Coefficient of the Intracellular Viral DNA

	Control Ad 12[a] (cpm)	Heat inactivated Ad 12[b] (cpm)
BHK cells (9.35×10^6 cells)		
Uptake of Ad 12	528	14,792
Label eluting into medium	150	4,700
Sedimentation coefficient of labeled DNA in cell	29 S (100%)	16.6 S (92%)
HEL cells (5×10^5 cells)		
Uptake of Ad 12	228	8,020
Label eluting into medium	0	3,100
Sedimentation coefficient of labeled DNA in cell	29 S (90%) 18.5 S (10%)	18.6 S (100%)
KB cells (8×10^5 cells)		
Uptake of Ad 12	1,852	5,880
Label eluting into medium	250	2,000
Sedimentation coefficient of labeled DNA in cell	> 45 S (1%) 29 S (94%) 14 S (4%)	17 S (94%)

[a] Infectivity titer: 1.7×10^{10} PFU/ml.

[b] Heated to 56°C for 30 min, infectivity titer: < 10 PFU/ml.

The effect of UV, formaldehyde and β-propiolactone inactivation of the virus were also studied. The Ad 12 inoculum was inactivated by dialysis against 1000 volumes of 1% formaldehyde or 4 g/l β-propiolactone in PBS at 4°C for 18 hr. This was followed by dialysis against four changes of 1000 volumes of PBS. A sample of Ad 12 suspended in 0.5 ml PBS was inactivated in 60 mm petri dishes by UV irradiation from a General Electric G15T8 germicidal UV lamp. Monolayers of BHK, KB, and HEL cells were inoculated with inactivated virus. The total intracellular DNA was extracted and analyzed 7 hr after infection. The results of these experiments are summarized in Table 21.

The DNA of the UV- and formaldehyde-inactivated Ad 12 has the same sedimentation pattern as the DNA from the control virus. However, 20 to 30% of the DNA from β-propiolactone treated Ad 12 was fragmented to pieces sedimenting about 21 S. Ultraviolet light which acts mainly on the viral DNA has no effect on the size distribution of the intracellular Ad 12 DNA. Formaldehyde and β-propiolactone which interact with both viral DNA and proteins inhibit the formation of the slowly sedimenting DNA.

7. Endonuclease activity of extracts from cells infected with adenovirus. The experiments presented show that adenovirus DNA in infected cells is eleaved to fragments which sediment at 18.6 S, and further suggest that these specific cleavages are made by an endonuclease. This endonuclease should be recoverable from extracts of infected cells.

a. A test of several methods of extracting cells. Several techniques of cell extraction were tested to find a method with maximum recovery of endonuclease activity and minimum contamination with cellular exonuclease. McAuslan et al. (1965) and Ledinko and Fong (1969) could find no altered exonuclease activity in cells infected with adenovirus. The results of this experiment are summarized in Table 22. Extraction with detergents or grinding with abrasives destroyed all endonuclease activity. Extraction by freezing and thawing, sonication, Dounce homogenization and lysis in distilled water yielded about the same amount of exonuclease activity. However, extracts made by the first two methods

Table 21

The Fate of Viral DNA in BHK, HEL, and KB Cells Inoculated with Adenovirus Type 12 Inactivated by Ultraviolet Light, Formaldehyde or β-Propiolactone

		% of intracellular DNA found as species which sediments at:		
	% uptake	> 45 S	29 S	< 25 S
Ad 12 inoculum				
control[a]			94	
UV inactivated[b]			91	
formaldehyde inactivated[c]			96	
β-propiolactone inactivated[d]			72	26
BHK				
control	.36	4	80	12
UV inactivated	.28	1	81	14
formaldehyde inactivated	.06		92	
β-propiolactone inactivated	.12		72	25
HEL				
control	.18	2	78	14
UV inactivated	.13	1	73	18
formaldehyde inactivated	.11		97	
β-propiolactone inactivated	.28		74	24
KB				
control	.23	4	83	12
UV inactivated	.12	2	76	19
formaldehyde inactivated	.08		90	3
β-propiolactone inactivated	.10		68	28

Infectivity titers:

[a] 4×10^9 PFU/ml

[b] 20 PFU/ml

[c] < 10 PFU

[d] < 10 PFU/ml

Table 22

Various Methods of Extracting Endonuclease from BHK Cells Infected with Adenovirus Type 12

Method of extraction	% of substrate acid soluble	Sedimentation coefficient of DNA substrate
Control - buffer only	0	29
Control - extract from uninfected cells[a]	4	29
Freeze - thaw three times	10	13
Sonication - 3 min at 2.2 amp	11	13.5
Grinding with alumina	0	29
Grinding with pyrex	0	29
Dounce homogenization - 25 strokes	8	17
Distilled water	11	17
0.1% SDS	0	27
0.1% Triton X 100, 0.001% spermine	7	31

[a] Extracted by freeze-thawing three times.

Cells were grown to confluent monolayers of about 5×10^7 cells per bottle. These cells were infected with 200 PFU of purified Ad 12 per cell. The inoculum was removed, the cells were washed and incubated in maintenance medium. At 10 hr after infection the cells were suspended at a final concentration of 5×10^7 cells/ml in 0.15 M KCl, 0.01 M tris-HCl buffer at pH 7.2. A 2 ml aliquot of the suspension was used to test each extraction method. The nuclease activity was assayed by adding 1.0 µg of Ad 12 DNA-^{3}H to 1 ml of extracted cells. After incubating at 37°C for 1 hr, the amount of radioactivity rendered acid soluble during incubation was determined. The DNA was extracted from the reaction mixture and the size of the DNA was analyzed.

listed formed DNA fragments sedimenting at 13 S, instead of 17 S, possibly due to increased activity of the endonuclease. The technique of freezing and thawing was selected for further experiments.

b. Endonuclease activity of cell extracts. The appearance of the endonuclease activity was determined in cell extracts of BHK, HEL and KB cells infected with Ad 12. Monolayers of these cells were infected with 60 PFU of Ad 12 per cell. Control cells were mock infected. The cells were suspended 12 hr after infection, washed, and resuspended in PBS at 5 x 10^6 cells/ml and then disrupted by freezing and thawing three times. The extracts were clarified at 100,000 g-hr and the supernatant fluid was used as the source of endonuclease. The results of experiments in which BHK and HEL cells were used are illustrated in Figure 14.

The extracts from uninfected HEL cells had no enconuclease or exonuclease activity and the Ad 12 DNA-^{3}H cosedimented with the marker. The extracts from uninfected BHK cells, however, hydrolyzed the Ad 12 DNA-^{3}H which caused an asymmetrical distribution of the label in neutral sucrose gradient analysis. Non-sedimenting, acid-soluble label appeared at the top of the gradient. This phenomenon is more apparent when the results of experiments with extracts from mock-infected BHK cells are compared to the results with extracts from mock-infected HEL cells. The hydrolysis of the Ad 12 DNA by extracts of uninfected cells is presumably caused by a BHK cellular exonuclease recently described by Koh et al. (1970). All cell cultures were free from Mycoplasma and thus the exonuclease described by Stock and Gentry (1969) associated with Mycoplasma hominis was not involved. However, the Ad 12 DNA-^{3}H incubated with extracts from the infected BHK cells is distributed more symmetrically than the Ad 12 DNA-^{3}H incubated with extracts from uninfected BHK cells. The sedimentation coefficient of the mode is decreased from 19.6 S to 16.0 S.

The results obtained with extracts from HEL cells are less complicated. The sedimentation coefficient of Ad 12 DNA-^{3}H incubated with extracts from infected HEL cells is decreased from 29 S to 18.7 S. The label is distributed symmetrically and non-sedimenting material is not found at the top of the gradients. Extracts from KB cells infected with Ad 2 and Ad 12 also

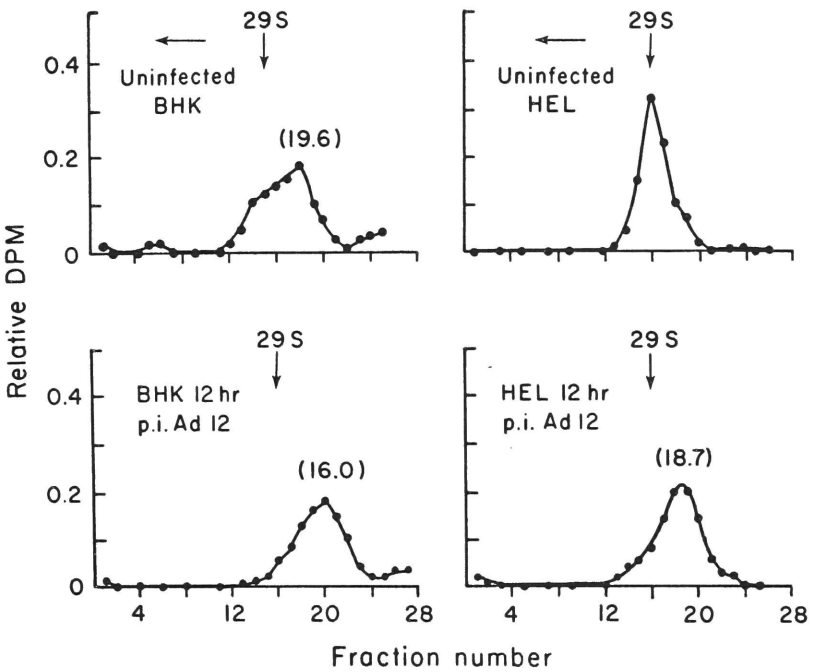

Figure 14: Endonuclease activity of BHK and HEL cells infected with adenovirus type 12. Cytoplasmic extracts were prepared from Ad 12 and mock-infected cells. Two micrograms of Ad 12 DNA-^{3}H were added to 0.5 ml of extract and incubated at 37°C for 60 min. The DNA was then extracted and the size of the DNA determined by zonal sedimentation on neutral sucrose density gradients.

hydrolyzed the Ad 12-^{3}H DNA. Thus when extracts from cells infected with Ad 2 or Ad 12 are incubated in vitro with adenovirus DNA, the adenovirus DNA is hydrolyzed to fragments of 16 to 19 S, a size similar to the one found in infected cells. Thus the results indicate that infection of the cells with adenovirus induces or activates an endonuclease within the cell.

c. Changes in the endonuclease activity during infection of cells with adenovirus. If the endonuclease was an enzyme coded for by the viral genome, it probably would have a distinct time point of appearance and would show a definite pattern of synthesis. The endonuclease could be either one of the early proteins which are synthesized about 6 hr after infection with a maximum concentration at 9 hr after infection and which disappears at 13 hr, or one of the late proteins which are synthesized about 10 hr after infection and reach a maximum at 12 to 16 hr after infection (Hayashi and Russell, 1968).

Petri plates with monolayers of BHK, HEL, and KB cells were infected with about 10^{3} PFU/cell of Ad 2 or Ad 12. The cell concentration was reduced to 10^{5} cells/ml at the time the cells were extracted and at this concentration, the exonuclease activity could not be detected. The cells were extracted at times as described below and the medium, the 100,000 X g supernatant fluid of the cytoplasmic extract and the resuspended pellet from the cytoplasmic extract were assayed for endonuclease activity with either 1 or 10 µg of Ad 12 DNA-^{3}H. After incubation at 37°C for 60 min, the DNA was extracted and the size of the DNA was determined by zonal sedimentation on neutral sucrose gradients. The results of experiments with supernatant fluids of BHK cells infected with Ad 2 or Ad 12 are shown in Figure 15.

The endonuclease activity of both Ad 2 and Ad 12 in BHK cells first appears at 4 hr after infection and reaches a maximum about 8 hr after infection. The BHK cells are productively infected with Ad 2 and in this system, the endonuclease activity of cell extracts increases sharply at 12 to 14 hr after infection. However, in BHK cells infected with Ad 12, there is no additional increase in endonuclease activity at 12 to 14 hr

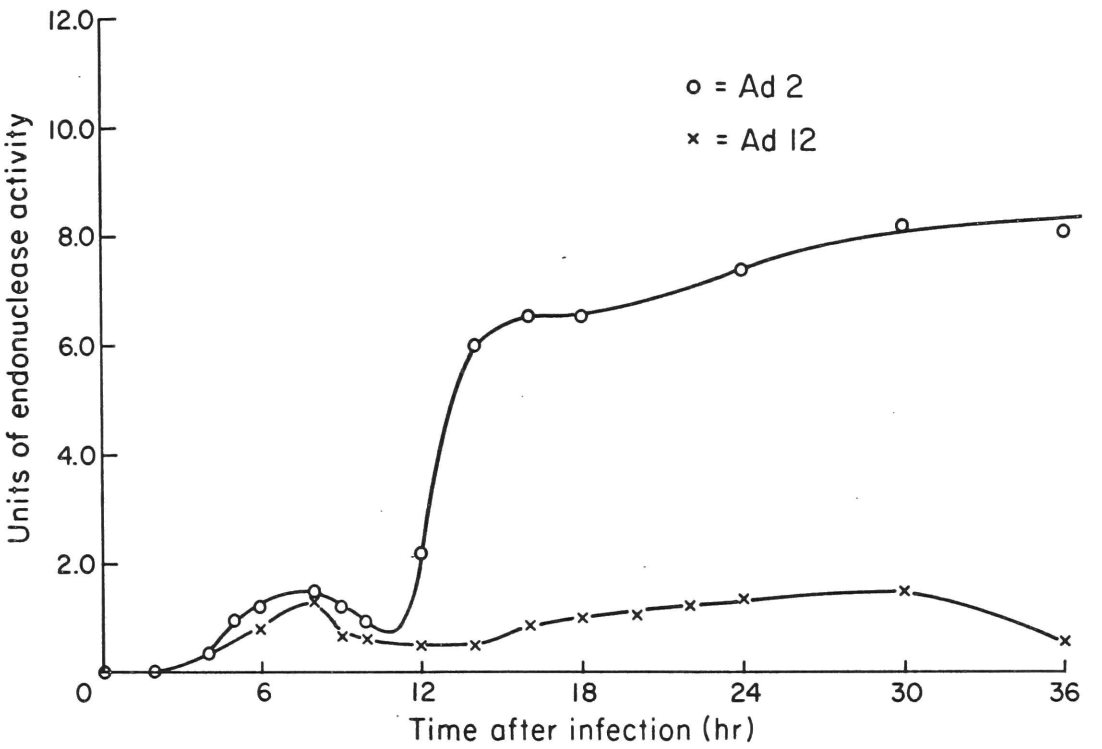

Figure 15: Endonuclease activity of the cytoplasmic supernatant fluid from BHK cells infected with adenovirus types 2 or 12. At various times after infection, the cells were extracted. The 100,000 X g supernatant fluid of the freeze-thaw extracted cells was assayed for endonuclease activity. One unit of activity is equal to the loss of 1 µg of intact Ad 12 DNA-^{3}H per hr.

and only a very slow increase in activity between 14 and 30 hr after infection. BHK cells are nonproductively infected with Ad 12. Thus the increase in endonuclease activity associated with the productive cycle is not seen in the nonproductive cycle. These results suggest that the endonuclease is a late or structural protein.

However, if the endonuclease was strictly a structural protein which is newly synthesized, there should be no endonuclease activity present before the beginning of DNA synthesis at 10 hr after infection. If the endonuclease is a structural protein, the low level of activity actually found before DNA synthesis may be due to the high multiplicity of infection used. The virion would carry the endonuclease molecules and could introduce them into the cells. The time of this early increase in activity corresponds well with the period of uncoating which might lead to a release of viral proteins into the cytoplasm. This interpretation would explain the endonuclease activity found early after infection and the endonuclease found late after infection would be newly synthesized.

d. Dependence of the endonuclease activity on multiplicity of infection. If the endonuclease found in extracts of cells between 4 and 10 hr after infection was indeed carried into the cells by the virions, which infect the cell, the endonuclease activity at this time should be proportional to the multiplicity of infection. Such a dependence should not be found for the increase in endonuclease activity which occurs late in productively infected cells, since this secondary increase is most likely due to newly synthesized enzyme. This hypothesis was tested in BHK cells infected with Ad 2 at multiplicities of infection which ranged from 0.027 to 14,000 PFU/cell. The endonuclease activity was assayed for each multiplicity used at 8, 16 and 24 hr after infection of BHK cells. The results of this experiment are listed in Table 23.

The endonuclease activity found in BHK cells 8 hr after infection with Ad 2 drops rapidly as the multiplicity of infection decreases. When 54 PFU/cell were used, only a trace of early endonuclease activity is detectable; however, the amount of endonuclease activity found at 24 hr

Table 23

Dependence of Endonuclease Activity
on the Multiplicity of Infection

Multiplicity of infection	Units of endonuclease activity[a]		
	8 hr p.i.	16 hr p.i.	24 hr p.i.
14,000	1.9	6.8	7.4
980	0.75	6.1	7.6
54	0.05	5.9	7.3
4	0.0	3.7	6.4
0.3	0.0	0.6	1.4
0.027	0.0	0.0	0.09

[a] One unit of enzyme corresponds to 1 μg Ad 2 DNA-^{3}H cleaved per hr by the supernatant fluid made from cells extracted by freezing and thawing.

after infection is not altered. The amount of endonuclease, presumably newly synthesized, found at 24 hr after infection declines only when less than 1 PFU/cell is used.

These data support the hypothesis that the endonuclease activity found early in the growth cycle of the virus is carried in to the cell by the virions and that the endonuclease activity found after DNA synthesis is associated with a late viral protein. There is an implicit assumption in this interpretation that the amount of endonuclease activity measured is directly proportional to the concentration of endonuclease in the extract. This premise has not been examined experimentally for the adenovirus endonuclease, but has been found valid for many other enzymes. Thus the amount of endonuclease released per virion cannot be quantitated from these data. The amount of endonuclease synthesized per virion cannot be determined either since the proportion of virions adsorbed from the inoculum and the residual amount of early endonuclease is unknown.

e. Endonuclease activity of cells infected with light inactivated adenovirus. The origin of the early and late endonuclease may also be traced if the viral genome is inactivated without damage to the viral proteins. This can be done if virus grown in the presence of 10 μg 5-bromodeoxyuridine per ml of medium is exposed to light. Monolayers of BHK and KB cells were infected with such inactivated virus preparations and the endonuclease activity was assayed at 8 hr and 24 hr after infection. The results of this experiment are listed in Table 24.

The Ad 2 labeled with 5-bromodeoxyuridine was rapidly inactivated by light with single hit kinetics. When BHK or KB cells were inoculated with partial or completely inactivated virus preparations, the amount of endonuclease activity found in cell extracts at 6 hr after infection was nearly constant between 1.6 to 2.3 units, regardless of the infectivity titer of the inoculum. Cells inoculated with inactivated Ad 2 contained no detectable endonuclease at 24 hr after infection.

These results clearly demonstrate that the endonuclease activity which is present early after infection is preformed and is a portion of the virion which infected the cell. Furthermore, these data indicate

Table 24

Endonuclease Activity in Cells Isolated with
5-Bromodeoxyuridine Labeled Adenovirus Type 2 Inactivated by Light

Exposure to light[a] (min)	Infectivity titer (PFU/ml)	Units of Endonuclease Activity			
		BHK cells[b]		KB cells[c]	
		6 hr p.i.	24 hr p.i.	6 hr p.i.	24 hr p.i.
0	3.4×10^{10}	2.1	8.4	2.3	10.4
1	9.4×10^{7}	1.9	1.4	2.1	1.7
2	2.8×10^{5}	2.3	0.21	1.9	0.24
3	9.2×10^{2}	1.7	0	1.9	0
4	5	1.9	0	1.7	0
5	< 1	1.7	0	1.7	0
10	< 1	1.6	0	1.5	0

[a] The Ad 2 sample in 1 ml PBS in a 60 mm petri dish on a white background was exposed to a 30 watt fluorescent light 4 cm away.

[b] 4.3×10^{6} cells/plate.

[c] 2.7×10^{6} cells/plate.

that the endonuclease which is present at 24 hr after infection is newly synthesized. These data also suggest that the endonuclease introduced by the virion is inactivated by 24 hr after infection, since there is no detectable activity at 24 hr after infection with inactivated virus.

If these interpretations are correct, one could postulate that the endonuclease found in BHK cells abortively infected with Ad 12 (see Fig. 15) could be newly synthesized, although the endonuclease is presumably a late viral protein. Doerfler (1969) has shown that the DNA of Ad 12 does not replicate in BHK cells, hence, late proteins should not be synthesized. However, Huebner et al. (1964a) and Berman and Rowe (1965) have identified fiber antigens in transformed hamster cells and Strohl et al. (1966) have presented evidence that small amounts of late Ad 12 proteins are synthesized in a few of the infected cells. The low level of endonuclease in the BHK cells infected with Ad 12 could be explained if the block for late protein synthesis is incomplete. Also, the mechanism which inactivates the early endonuclease may be blocked and the low level of late endonuclease still represents preformed protein from the virion.

All of the evidence presented indicates that the virion carries the endonuclease and that the viral genome is responsible for the de novo synthesis of the endonuclease in the cell. The endonuclease presumably is a late protein, possibly a capsid protein.

8. Endonuclease activity associated with adenovirions. The evidence presented indicates that the adenovirions which infect the cells contained an endonuclease. This hypothesis was tested directly when purified virions were assayed for endonuclease activity in vitro.

a. In vitro assay of the endonuclease activity associated with adenovirions. Experimental evidence described in Section IV. B.6. indicate that the DNA inside the virion is hydrolyzed to 18 S fragments during incubation at 56°C for 30 min. This DNA product sedimented homogeneously in neutral and alkaline sucrose density gradients. Intact Ad 12-^{3}H was incubated with purified Ad 12 virions or PBS. This will determine whether the endonuclease can hydrolyze the DNA in solution as well as the DNA within the virion. Figure 16 illustrates the results of this experiment.

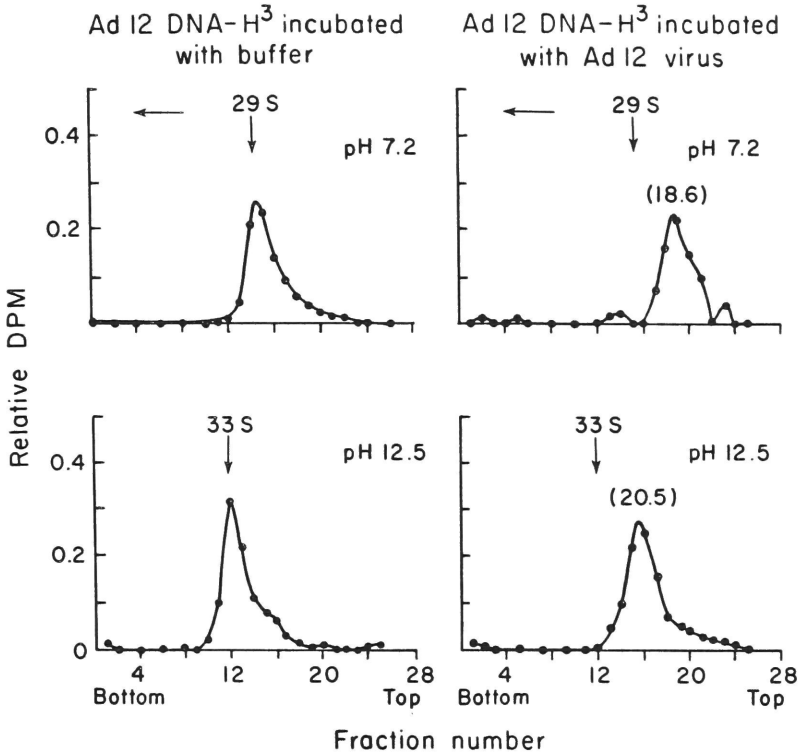

Figure 16: Endonuclease activity of purified virions of adenovirus type 12. About 100 µg of purified Ad 12 virions were added to 10 µg of Ad 12 DNA-^{3}H. This mixture was incubated at 37°C for 1 hr. The control mixtures contained only Ad 12 DNA-^{3}H and buffer. After incubation, the DNA was extracted and the size of the DNA was determined in neutral and alkaline sucrose density gradients.

The DNA incubated with PBS cosediments with the marker and is homogeneous in both neutral and alkaline gradients. However, the Ad 12 DNA incubated with the purified Ad 12 virions sediments as a homogeneous species at 18.6 S in neutral and 20.5 S in alkaline gradients. Exonuclease activity is not present, since label which does not sediment is not detectable. Labeled Ad 2 DNA incubated with either Ad 12 or Ad 2 virions is also hydrolyzed to 18.6 S DNA. Thus the endonuclease is clearly associated with the purified virions. These data do not distinguish whether the endonuclease is a structural component of the virion or a cellular or viral protein adsorbed to the virion.

b. Characteristics of the DNA product. It must be stressed that when adenovirus DNA is the substrate, the DNA product of this endonuclease reaction is homogeneous and apparently has a specific size. Thus the endonuclease hydrolyzes the DNA into fragments which are an integral fraction of the intact adenovirus DNA. The molecular weight of these fragments can be calculated from the sedimentation coefficient. The molecular weight of the fragments is about 5 million Daltons, i.e. the DNA fragments generated by incubation with the virion endonuclease have one-fourth the molecular weight of the intact DNA. Since 4 pieces, each 5.4 million Daltons, are formed from each molecule of Ad 2 (24 million Daltons), the site of the endonuclease attack may coincide with the guanine-cytosine rich regions of the Ad 2 DNA molecule (see Section I. C.4.).

The molecular weight of the single-stranded DNA found in alkaline gradients is one-fourth the molecular weight of the intact denatured DNA. If the endonuclease generated random single-strand breaks, the size of the denatured DNA after the reaction should be very small. However, since the size of the denatured DNA fragments corresponds with the size of the native DNA fragments, only double-stranded scissions can occur and these scissions probably are at specific sites.

c. The endonuclease is a virus-specific component of the virion. The endonuclease associated with the virion could be a cell-coded protein adsorbed onto the virion. This possibility was investigated in three experiments: 1) infected cells and purified virions were assayed for

cellular enzymes, 2) the endonuclease activity was correlated with virion concentration during purification, and 3) the endonuclease was inactivated with virus-specific antiserum.

1) Other enzymes associated with adenovirions. Purified virions and extracts from infected cells were assayed for endonuclease, exonuclease, protease and ATPase activity. The results of these determinations are listed in Table 25. Clearly, the cell extracts had all four of these enzyme activities but the only enzyme activity associated with the purified virion was the endonuclease.

Enzymes found in the infected cell are not associated with the purified virion. These data suggest it is unlikely that the endonuclease is a cellular enzyme associated with the Ad 2 virion by nonspecific adsorption.

2) Endonuclease activity during purification of the virions. Two parameters, infectivity titer and endonuclease activity, of homogenates of KB cells infected with Ad 2 were compared with these parameters in virus preparations during various steps of the purification procedure. These data are found in Table 26.

There is a large amount of endonuclease associated with the homogenate from KB cells infected with Ad 2. Earlier data indicated that the endonuclease was a structural protein. Ginsberg and Dixon (1959) and Green (1962) showed that only 10 to 15% of the structural proteins synthesized in the infected cells were incorporated into virus particles. Compared to the viral infectivity titer, there is a great excess of endonuclease in the cell homogenates. However, after the second CsCl purification, the ratio of infectivity to endonuclease is relatively constant at 2.6 to 2.7 x 10^9 PFU per unit of endonuclease activity. Therefore, the endonuclease activity must be firmly found to the virion. The infectivity and endonuclease activity in six other purified preparations of Ad 2 were determined and the ratios were 2.4 to 2.8 x 10^9 PFU per unit of endonuclease. These findings further support the hypothesis that the endonuclease is a component of the virion.

Table 25

Enzymes in Extracts of KB Cells
Infected with Adenovirus Type 2
and in Preparations of Purified Adenovirions

Preparation	Enzyme assayed			
	Endonuclease[a]	Exonuclease[b]	Protease[c]	ATPase[d]
Extract of KB cells infected with Ad 2				
1. Extract[e]	+	+	+	+
2. Heat inactivated extract[f]	+	-	±	-
3. Boiled extract[g]	-	-	-	-
Purified Ad 2 virions				
1. Virions[h]	+	-	-	-
2. Heat inactivated virions[f]	+	-	-	-
3. Boiled virions[g]	-	-	-	-

[a] Determined by the method described in Section III. A.8.

[b] Determined by the release of acid soluble radioactivity from DNA-^{3}H. The reaction mixture contained 10 mM tris-HCl at pH 7.5, 300 mM KCl, 40 mM $MgSO_4$, 20 mM β-mercaptoethanol, 2 µg Ad 2 DNA-^{3}H (about 3 x 10^4 cpm).

[c] Determined by the release of acid soluble radioactivity from protein-^{3}H. The reaction mixture contained: 10 mM tris HCl at pH 7.5, 10 µg KB cell protein-^{3}H (about 6 x 10^4 cpm).

[d] Determined by the release of ^{32}p from ATP-γ-^{32}p. The reaction mixture contained 10 mM tris at pH 7.5, 10 mM $MgCl_2$, 200 mM KCl, ATP-γ-^{32}p (10^4 cpm).

[e] A suspension of 5 x 10^6 KB cells/ml was frozen and thawed three times and the 100,000 X g supernatant was used.

[f] The preparation was heated to 56°C for 30 min.

[g] The preparation was heated in boiling water bath for 10 min.

[h] The virions were purified by three CsCl equilibrium sedimentation gradients and dialyzed for use.

Table 26

Infectivity Titer and Endonuclease Activity
of Virions of Adenovirus Type 2
During Various Steps of Purification

Step	Infectivity Titer PFU/ml[a] ($\times 10^9$)	Endonuclease Activity Units/ml[a]	Ratio PFU/unit ($\times 10^{-9}$)
Homogenate of Cells infected with Ad 2	1.8	140.0	.013
CsCl I[b]	3.4	9.4	.36
CsCl II	17.	6.3	2.70
CsCl III	67	25.0	2.68
CsCl IV	56	21.4	2.61

[a] Mean of three determinations.

[b] After each CsCl equilibrium sedimentation, a sample of virus was dialyzed and assayed.

3) Inhibition of the adenovirion endonuclease by antiserum. Should the endonuclease be a cell-coded enzyme adsorbed onto the virion, the endonuclease associated with Ad 2 and Ad 12 grown in KB cells would be antigenically identical.

The nucleases of specific neutralizing antiserum for Ad 2 and Ad 12 (see Section II. H.) were heat inactivated at 70°C for 5 min as suggested by Lehman and Nussbaum (1964). The specificity and virus neutralizing activity of the antisera were then determined. Purified Ad 2 and Ad 12 were reacted with these specific neutralizing antisera at 37°C for 30 min and then assayed for endonuclease activity. These data are found in Table 27.

The antiserum against Ad 2 and Ad 12 specifically inactivates the endonuclease associated with the homologous virion. These data imply that the endonuclease of Ad 2 and Ad 12 are antigenically different although both viruses are grown in KB cells. Furthermore, the antiserum also inactivated the endonuclease activity of Ad 2 produced in HeLa and BHK cells. Hence, the structure of the endonuclease is coded for by the viral genome and not the cellular genome.

d. Reaction conditions.

1) pH optimum. The endonuclease associated with Ad 2 virions is completely inhibited in buffers with pH less than 5 and greater than 9.0. There is a sharp maximum of activity between pH 7.0 and 7.5. Thus pH 7.2 was selected for all assays of endonuclease activity associated with virions.

2) Supplemental ions. The endonuclease was assayed in PBS or 0.01 M Tris-HCl at pH 7.2. Even when 0.001 M EDTA is added to the tris buffer, the endonuclease activity is present. Thus divalent ions do not appear essential or they cannot be removed from the virion by EDTA. Furthermore, when the reaction mixture is supplemented with calcium, magnesium or manganese, there is no apparent effect on the size of the end product or the rate of hydrolysis.

Table 27

Inhibition of Adenovirus Endonuclease by Specific Antiserum

Antiserum	Neutralization titer[a]	Percent inhibition of endonuclease associated with:	
		Ad 12 virion	Ad 2 virion
Control[b]	0	0	0
Anti-Ad 2	1:64	3	97
Anti-Ad 12	1:32	94	6

[a] After heat inactivation of nuclease activity at 70°C for 5 min.

[b] Serum from the rabbit before immunization.

3) Inhibitors. The endonuclease activity of the virion is inhibited by 1 mM β-mercaptoethanol, and 1 μg p-chloromercuribenzoate/ml, the endonuclease was inactivated by phenol, 0.5% SDS, 2 M CsCl, 1 M urea and boiling. The endonuclease can be readily digested by pronase and trypsin.

4) Age of adenovirions. After the adenovirions were purified and dialyzed into PBS, there was a slow decline in the endonuclease activity. Seven days after dialysis little or no endonuclease activity remained in the preparation. During this time, the virions were altered structurally and became susceptible to digestion by proteolytic enzymes. This loss of endonuclease activity could not be prevented by adding 10% glycerol, 1% bovine serum albumin, or medium to the virus suspension. This suggested that the virion had undergone a conformational change. Thus only freshly purified and dialyzed adenovirions could be used as a source of endonuclease.

e. Heat stability of the endonuclease associated with adenovirions. Aliquots of purified Ad 12 virions were heated at 56°C for 30 min and fractionated by centrifugation into a 100,000 X g supernatant fluid and pellet. Purified Ad 12 virions were also heated in a boiling water bath for 10 min. These preparations were then assayed for endonuclease activity by incubation at 37°C with 1 μg of Ad 12 DNA-^{3}H for 1 hr. The DNA was extracted and was analyzed in neutral sucrose gradients. The results of these experiments are illustrated in Figure 17.

These data show that the endonuclease is not destroyed by heating to 56°C for 30 min, but is inactivated by boiling. The endonuclease activity of unheated Ad 12 sediments with the virion and is found only in the viral pellet. After heating the Ad 12 virions to 56°C for 30 min, a large proportion of the endonuclease is found in the pellet; however, there is still a large amount of endonuclease in the supernatant fluid. Russell et al. (1967b) demonstrated that when Ad 5 is heated to 56°C the penton is released with its surrounding 5 hexons from the vertices of the icosahedron. Thus the pentons or the hexons may contain the endonuclease, although internal core proteins or adsorbed proteins may also be released during this procedure.

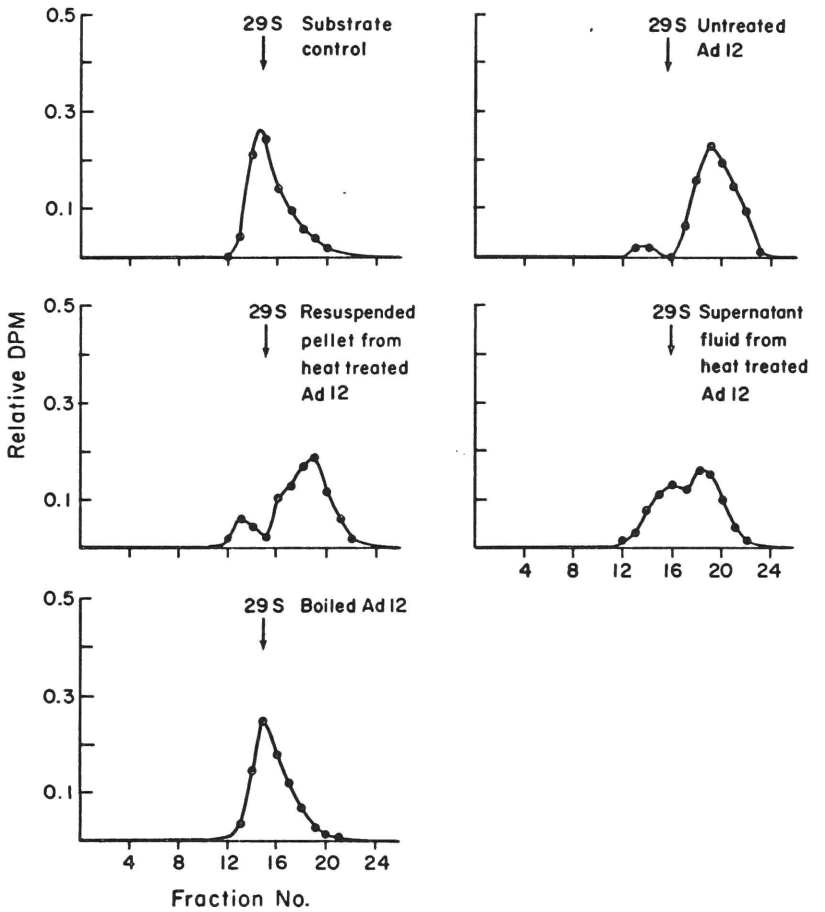

Figure 17: Heat stability of endonuclease associated with virions of adenovirus type 12. After aliquots of Ad 12 were heated to 56°C for 30 min, the preparations were incubated with 1 μg Ad 12 DNA-^{3}H for 1 hr at 37°C. The DNA was extracted and analyzed in neutral sucrose density gradients.

f. Substrate specificity of the endonuclease associated with the adenovirion. About 25 μg of purified Ad 2 virions were incubated for 1 hr with DNA extracted from Ad 2, Ad 12, KB, BHK, and L cells. The cellular DNA had been prepared as a species which sedimented homogeneously by zonal sedimentation. After incubation, the DNA was extracted and was analyzed on neutral sucrose density gradients. The data from this experiment are listed in Table 28.

The endonuclease hydrolyzes each of the DNA substrates tested; however, only Ad 2 and Ad 12 DNA are cleaved to fragments which sediment homogeneously and are a specific size. Preparations of DNA from KB, BHK and L cells with a uniform size are also hydrolyzed but the size of the product is very heterogeneous.

g. Endonuclease activity of incomplete adenovirus particles. Smith (1965) described incomplete adenovirus particles which are morphologically intact but have a lower buoyant density in CsCl density gradients. Burlingham and Doerfler (1969) described incomplete Ad 2 and Ad 12 particles with a buoyant density higher than that expected for empty capsids. Maizel et al. (1968a,b) examined the proteins of these incomplete particles by polyacrylamide gel electrophoresis. The polypeptide pattern was identical to that of intact virions, except that one of the polypeptides was present in smaller amounts. Thus incomplete adenovirus particles apparently have all of the capsid proteins of the complete virion.

Incomplete adenovirus particles were assayed for endonuclease activity under the same conditions as used for complete adenovirions. The incomplete virus particles of Ad 2 and Ad 12 hydrolyzed Ad 2 DNA-^{3}H to fragments of 17 to 19 S. Thus the incomplete adenovirus particles, which contain all of the capsid proteins of adenovirions, also have the endonuclease activity.

The experimental evidence presented in this section documents the conclusion that an endonuclease activity is associated with complete and incomplete virus particles of Ad 2 and Ad 12. The size of the cleavage product, when viral DNA is the substrate, is identical with the size of viral DNA fragments found in cells infected with Ad 2 or Ad 12. The

Table 28

Substrate Specificity of the Endonuclease
Associated with Virions of Adenovirus Type 2

DNA substrate extracted from:	Sedimentation coefficient of native DNA incubated with:		
	Buffer	Boiled Ad 2 virions	Ad 2 virions
Ad 2	32.7	31.6	17.6
Ad 12	28.7	28.5	19.2
KB cells	38.0	37.0	7 to 12
BHK cells	35.7	37.6	4 to 10
BHK cells	23.5	26.2	3 to 15
L cells	47	46	4 to 20
L cells	34.0	34.2	3 to 19
L cells	9.22	8.9	3 to 7.8

endonuclease may reside in one of the capsid proteins. The endonuclease is heat stable, has a pH optimum of 7.0 to 7.5, and has no divalent ion requirements. The endonuclease can be digested by pronase and trypsin. The endonuclease activity is inhibited by β-mercaptoethanol, p-chloromercuribenzoate, and by high ionic strength. The endonuclease associated with adenovirions grown in KB, HeLa or BHK cells is specifically inactivated by homologous antiserum. The latter finding suggests that the endonuclease is virus coded.

9. Endonuclease associated with the penton subunits of adenovirions. The data from experiments on the synthesis of the endonuclease activity in infected cells and the association of the activity with the virion suggest that the endonuclease is associated with capsid structures. Each capsid structure is composed of one or more unique polypeptides (see Section I. C.2 and 3). The purified capsid subunits were assayed for endonuclease activity.

a. Endonuclease activity of virion subunits. Hexon, penton, consisting of a penton base and a fiber, and fiber subunits were assayed for endonuclease activity. Each of the subunits had been isolated as morphologically intact subunits. The results of these experiments are shown in Figure 18.

Only the penton subunits have the endonuclease activity and hydrolyze the DNA to fragments of about 1 million Daltons. The hexon and fiber subunits at 100 times the relative concentration of penton subunits had no activity. Analysis of the reaction products by zonal sedimentation in alkaline sucrose density gradients revealed that the fiber and hexon subunits do not increase the number of single-strand breaks in Ad 2 DNA. Thus the endonuclease activity is associated with the penton subunit. Since the fibers alone do not hydrolyze the Ad 2 DNA, the penton base probably is the endonuclease.

b. Endonuclease activity of penton treated with trypsin. When the penton was digested with trypsin, the penton base was selectively destroyed (Pettersson and Höglund, 1969). The fiber was much more resistant to digestion with trypsin than the penton base. The penton was digested with

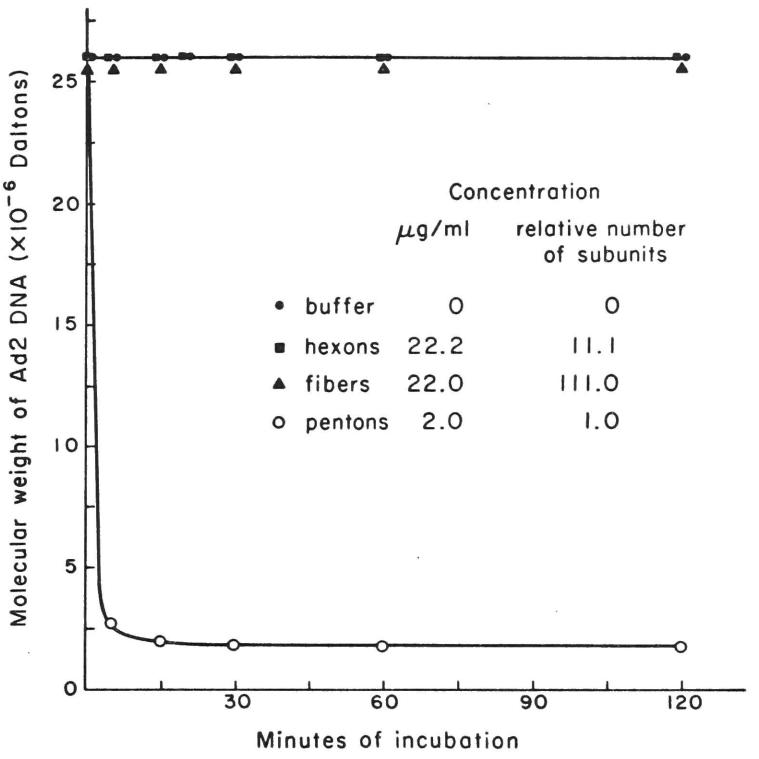

Figure 18: Endonuclease activity of structural subunits from adenovirus type 2. Each of the purified subunits was incubated with 5 μg of Ad 2 DNA-^{3}H at 37°C for 2 hr. Aliquots were taken at intervals, the DNA was extracted and was analyzed in neutral sucrose density gradients.

0.025 mg trypsin/ml and 0.25 mg trypsin/ml and assayed for endonuclease activity. The cytopathic titer and amount of penton base antigen present in this penton preparation had been determined by Pettersson.

The data shown in Table 29 demonstrate that digestion of the penton with 0.025 mg trypsin/ml destroys the endonuclease and the cytopathic effect, whereas the penton base antigen remains intact. When 2.5 mg trypsin/ml are used, the penton base antigen is reduced. These results indicate that the penton base and not the fiber is the endonuclease and the endonuclease may be involved in the cytopathic effect of the penton and the virion.

c. Competition between fiber or hexon and penton for DNA. The fiber is a part of the penton, but the isolated fibers do not have endonuclease activity. Do the fibers then participate in the endonuclease reaction? Levine and Ginsberg (1967a,b) have shown that purified fiber and hexon from cells infected with Ad 5 bind to cellular and viral DNA, and interfere with DNA- and RNA-polymerase activity in vitro. The degree of competition between fiber or hexon and the penton endonuclease for the viral DNA was determined. Various amounts of fiber or hexon were preincubated with 1 µg of Ad 2 DNA-^{3}H, then 1 µg of Ad 2 penton was added to the incubation mixture and the size of the DNA was determined at various times during the incubation. The results are illustrated in Figure 19.

Even high ratios of fiber or hexon to penton do not influence the initial rate of hydrolysis nor the final size of the DNA substrate. Fibers labeled with amino acids-^{3}H are bound to Ad 2 DNA before, during and after the hydrolysis. These findings support the conclusion that the penton base alone is the endonuclease and that the fiber does not participate in the endonuclease reaction.

d. Specific activity of endonuclease during purification of penton. The penton preparations used in all experiments are pure, as determined by four different physical techniques. The conclusion that the endonuclease does not represent a KB cell protein which contaminates the penton preparations was supported further by assaying for endonuclease activity at two

Table 29

Endonuclease Activity of Penton Treated with Trypsin

Trypsin (mg/ml)	% of Endonuclease activity	titer of cytopathic effect[a]	% of penton base antigen[a]
none	100	16,000	100
0.025	0	< 100	100
2.5	0	< 100	16

[a] Determined by U. Pettersson.

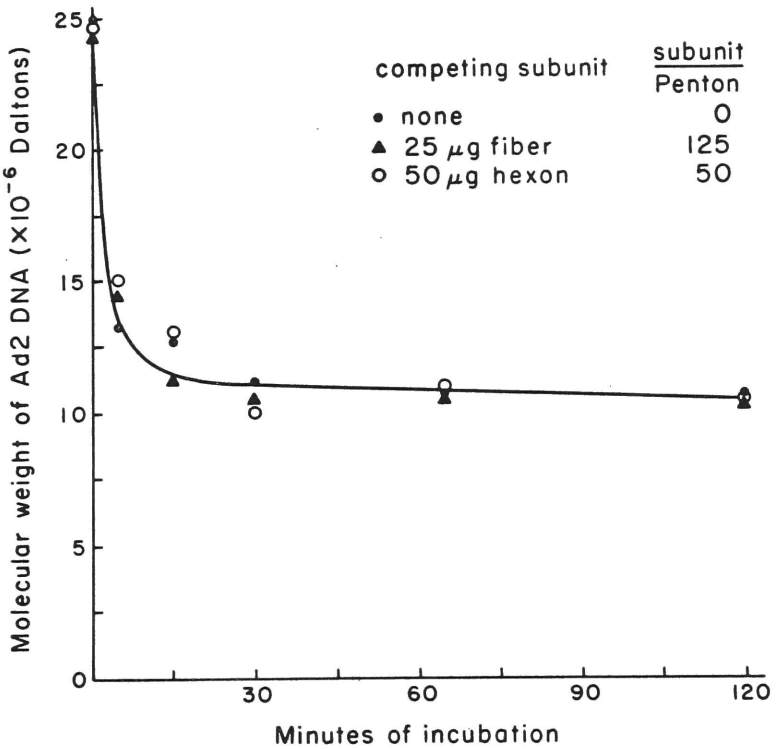

Figure 19: Competition between capsid subunits for adenovirus type 2 DNA. Various amounts of fiber or hexon were preincubated with 1 μg of Ad 2 DNA-^{3}H, then 1 μg of Ad 2 penton was added to the mixture. Aliquots were taken at intervals during incubation, the DNA was extracted and was analyzed in neutral sucrose gradients.

stages of purification. The total concentration of protein and the amount of penton in each preparation were determined by Philipson. The partially purified preparation of penton from the agarose column contained 1.75 mg protein, 1.1 mg penton and had 1100 units of endonuclease activity. The partially purified preparation was 63% penton and had 1.00 units of endonuclease activity per microgram penton protein. The highly purified preparation after preparative polyacrylamide gel electrophoresis contained 0.059 mg protein, 0.059 mg penton and had 65 units of endonuclease activity; i.e., a specific activity of 1.10 units of endonuclease activity per microgram penton protein. Since the specific activities of the partially and highly purified penton preparations are identical, it is unlikely that the endonuclease activity is due to a contaminant.

e. Inhibition of the endonuclease by antiserum. The data in Table 27 demonstrate that the endonuclease activity of the virion is specifically inhibited by homologous but not by heterologous antiserum. The anti-Ad 2 serum inhibited the endonuclease activity associated with the penton 100% but the anti-Ad 12 serum had no effect. Furthermore, antiserum prepared against highly purified Ad 2 penton neutralizes the endonuclease activity of Ad 2 virions but not of Ad 12 virions. These data, together with the studies of antiserum inhibition of the endonuclease activity associated with adenovirions (see Section IV, B.8.c.3) imply that the endonuclease of Ad 2 and Ad 12 are antigenically different although both viruses were grown in KB cells. This suggests that the structure of the endonuclease is coded for by the viral genome and not the cell genome.

All of the evidence presented supports the claim that the peptides of the penton base act as both an endonuclease and a structural component of the capsid.

f. Endonuclease reaction conditions. As a prelude to a study of the mechanism of action, the optimal reaction conditions for the endonuclease were defined.

1) Optimum pH. The pH optimum was determined with Tris-HCl buffer at pH values between 4.0 and 10.0. The data are shown in Figure 20.

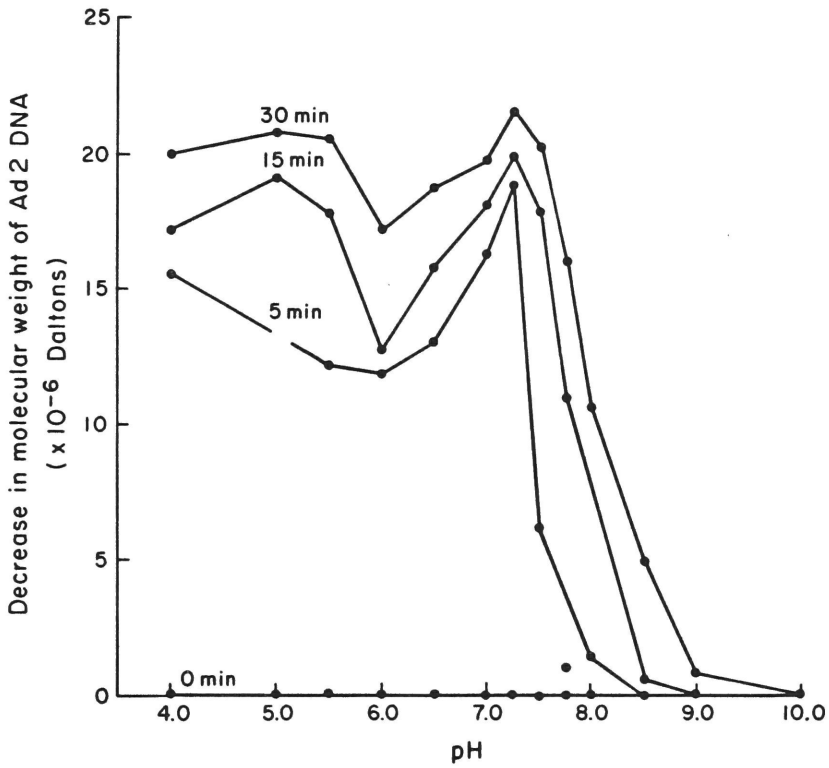

Figure 20: Optimum pH for the endonuclease reaction. Each reaction mixture contained 1 µg Ad 2 DNA-^{3}H, 1 µg Ad 2 penton, 10 mM tris-HCl buffer at pH values as indicated, 100 mM NaCl and 2 mM $MgCl_2$. The reaction mixtures were incubated at 37°C. At 5, 15 and 30 min, samples were removed, the DNA was extracted and the size of the DNA was determined.

The maximal rate of hydrolysis occurs at 7.25; however, a second optimum is present between pH 4.0 and 5.5. The endonuclease is markedly inhibited at pH 8.0 and completely inhibited at pH 9.0. The pH optimum for endonuclease associated with the virion is 7.0 to 7.5. For the standard assay buffer, pH 7.2 was selected.

2) Divalent ions. The requirements for divalent ions was studied, particularly for Mn^{++}, Mg^{++} and Ca^{++}. These data are found in Figure 21. The penton endonuclease is inactive without divalent ions. With Mn^{++} the rate of the reaction is the highest and very small fragments of DNA are formed. When 10 mM Mn^{++} is present, hydrolysis takes place even at 0°C. With Mg^{++} the rate of the reaction is rapid and the size of the DNA fragment is similar to that found in infected cells. When Ca^{++} is used, there is essentially no hydrolysis. However, 1 mM Ca^{++} does not inhibit the reaction supplemented with 1 mM Mg^{++}. For the standard assay buffer, 2 mM $MgCl_2$ was selected. The endonuclease associated with the virion apparently does not require divalent ions although the reaction is normally incubated in buffer with 2 mM Mg^{++}.

3) Tonicity. The effect of salt concentration on the penton endonuclease was determined by using NaCl at various concentrations. These data are found in Figure 22. The optimum activity is at a NaCl concentration of 0.02 M. The endonuclease activity is nearly constant at NaCl concentrations between 0.01 and 0.2 M. However, the reaction is completely inhibited by NaCl concentrations greater than 0.5 M. The endonuclease activity of the virion was also inhibited by high salt concentrations. The standard assay buffer contained 100 mM NaCl. This adjusted the reaction conditions to near physiological conditions.

4) Sodium/potassium ratio. The effect of the sodium/potassium ratio on the endonuclease activity was studied (Figure 23). Variation of the ratio of sodium to potassium has no effect on the activity of the endonuclease. The standard assay buffer does not contain potassium ions.

5) Temperature. The effect of temperature on the penton endonuclease was determined. These data are listed in Table 30. The change

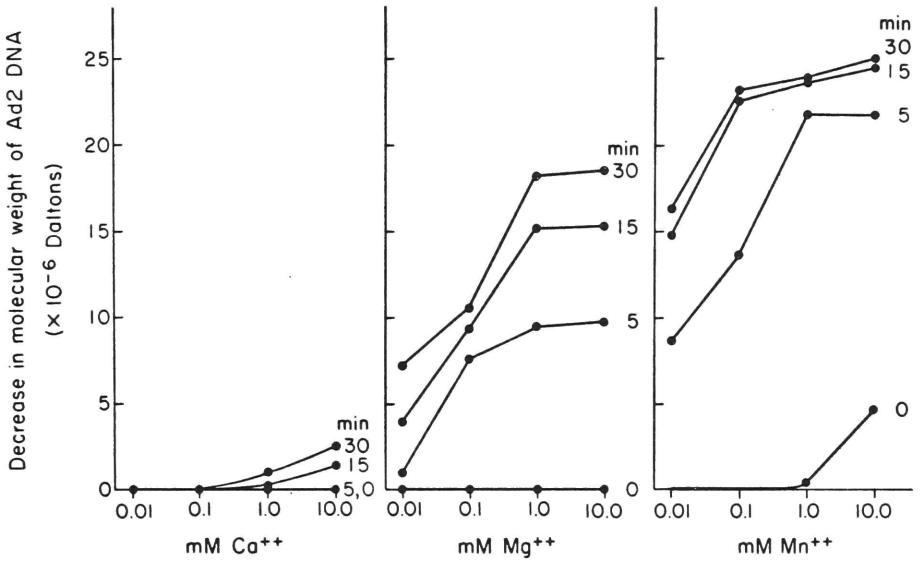

Figure 21: Requirement of the endonuclease reaction for divalent ions. The reaction mixture contained 1 μg Ad 2 DNA-^{3}H, 1 μg Ad 2 penton, 10 mM tris-HCl buffer at pH 7.2, 100 mM NaCl plus various amounts of either Ca^{++}, Mg^{++}, or Mn^{++}. At 5, 15 and 30 min after the reaction was started, samples were removed, the DNA was extracted and was analyzed in neutral sucrose gradients.

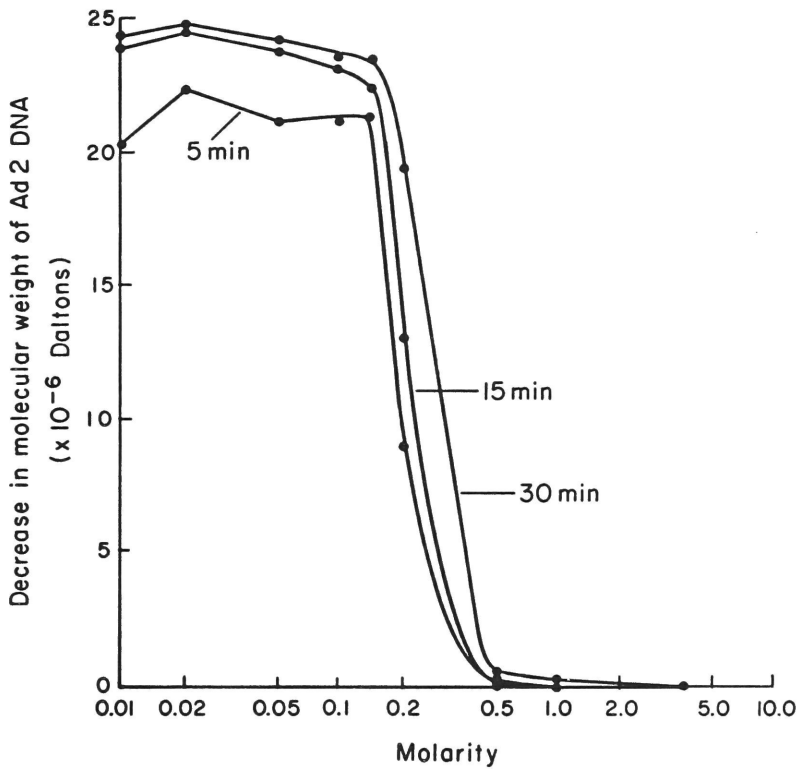

Figure 22: Effect of sodium chloride concentration on the penton endonuclease. Each reaction mixture contained 1 µg Ad 2 DNA-^{3}H, 1 µg Ad 2 penton, 10 mM tris-HCl buffer at pH 7.2, 2 mM $MgCl_2$ and various concentrations of NaCl. At 5, 15 and 30 min of incubation, samples were taken, the DNA was extracted and was analyzed in neutral sucrose gradients.

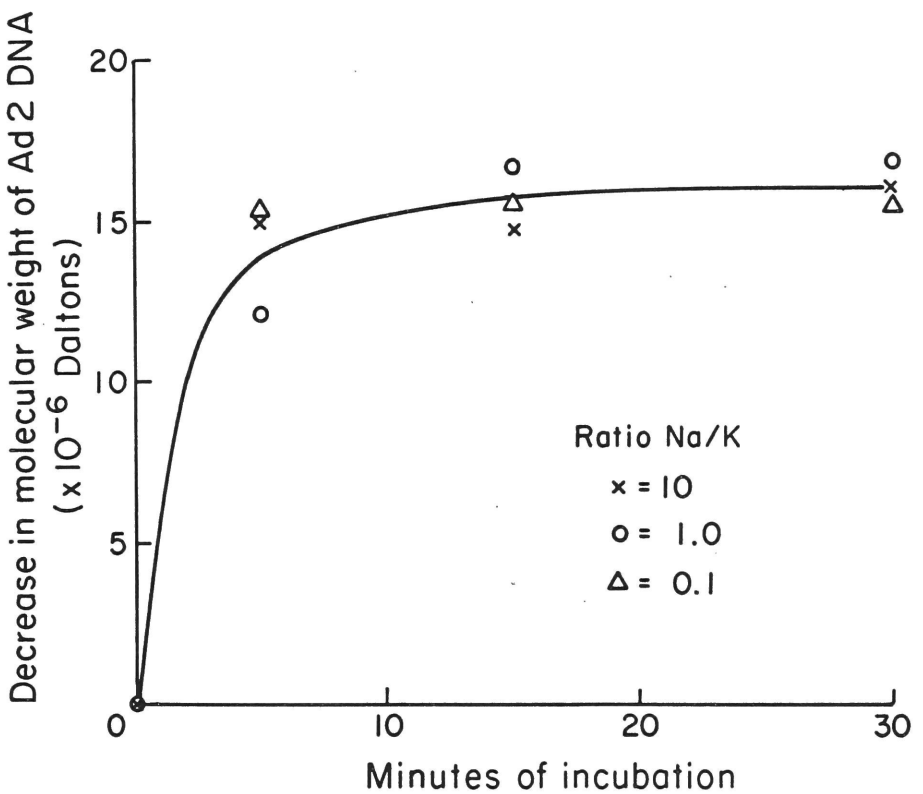

Figure 23: Effect of the sodium/potassium ratio on the activity of the penton endonuclease. The reaction mixture contained 1 µg Ad 2 DNA-^{3}H, 1 µg Ad 2 penton, 10 mM tris-HCl buffer at pH 7.2, 2 mM $MgCl_2$ and 100 mM NaCl or KCl in various ratios. The reaction mixture was incubated at 37°C and samples were taken at 5, 15 and 30 min, the DNA was extracted and analyzed in neutral sucrose gradients.

Table 30

Effect of Temperature on the Rate of Hydrolysis by the Adenovirus Type 2 Penton[a]

Temperature (°C)	Initial rate of hydrolysis (cleavages/penton/min)
0	0.016
10	0.33
20	0.66
30	1.4
40	3.5
60	10.0

[a] The reaction mixture contained 10 µg Ad 2 DNA-^{3}H, 1 µg Ad 2 penton, 100 mM NaCl, 10 mM tris-HCl, and 2 mM $MgCl_2$. The reaction mixtures were incubated at temperatures as indicated. Samples were taken at appropriate times, the DNA was extracted and was analyzed in neutral sucrose gradients.

in rate of the reaction increases until 30 to 40°C and then declines. The activation energy, calculated from the Arrhenius equation, for the hydrolysis of Ad 2 DNA by the penton endonuclease is 12,045 cal/mol. The standard temperature for incubation was 37°C.

6) Enzyme concentration.

a) Effect of penton concentration. The effect of penton concentration on the reaction was determined. The penton concentration was varied and the amount of substrate DNA remained constant. The data from this experiment are seen in Figure 24. Once a concentration of penton greater than 0.1 μg/ml is reached, the endonuclease is active. The DNA substrate decreases in size with only 0.1 μg/ml; however, apparently only small terminal fragments are removed. The higher the concentration of penton, the smaller the size of the DNA end product. In fact, when the DNA end product has a molecular weight of less than 5 million Daltons there is a linear relationship between the size of the DNA and the concentration of penton.

There is little change in the size of the DNA after the first 15 min of the reaction. These findings may be explained by assuming saturation of preferred reaction sites on the DNA substrate at a particular penton concentration. Alternatively, the endonuclease or DNA might no longer be reactive after the first 15 min of the reaction.

b) Effect of adding new substrate. After the size of the DNA in the reaction mixture which contained Ad 2 penton and Ad 2 DNA-^{3}H became constant, a second sample of Ad 2 DNA-^{3}H was added. The newly added DNA was rapidly hydrolyzed to the same final size as the original DNA which had been present from the beginning of the reaction (Figure 25). Thus the pentons are still active after 30 min of incubation with Ad 2 DNA.

c) Effect of adding new enzyme. After the endonuclease reaction reached equilibrium, a second sample of penton was added. The results of this experiment are seen in Figure 26. During the first 60 min of incubation the DNA was hydrolyzed to fragments of 2 million Daltons. When the second sample of penton was added, these fragments were

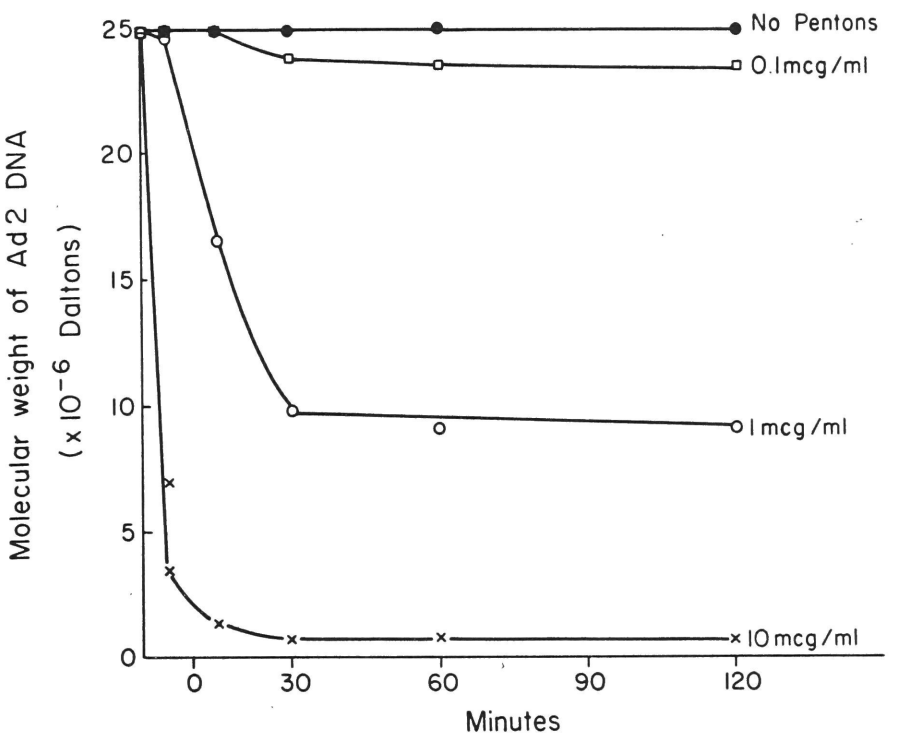

Figure 24: Effect of penton concentration on the size of adenovirus type 2 DNA. The reaction mixture contained 10 µg Ad 2 DNA-^{3}H, various amounts of penton, 100 mM NaCl, 10 mM tris-HCl buffer at pH 7.2, and 2 mM $MgCl_2$. At intervals during incubation, samples were removed, the DNA was extracted and was analyzed in neutral sucrose gradients.

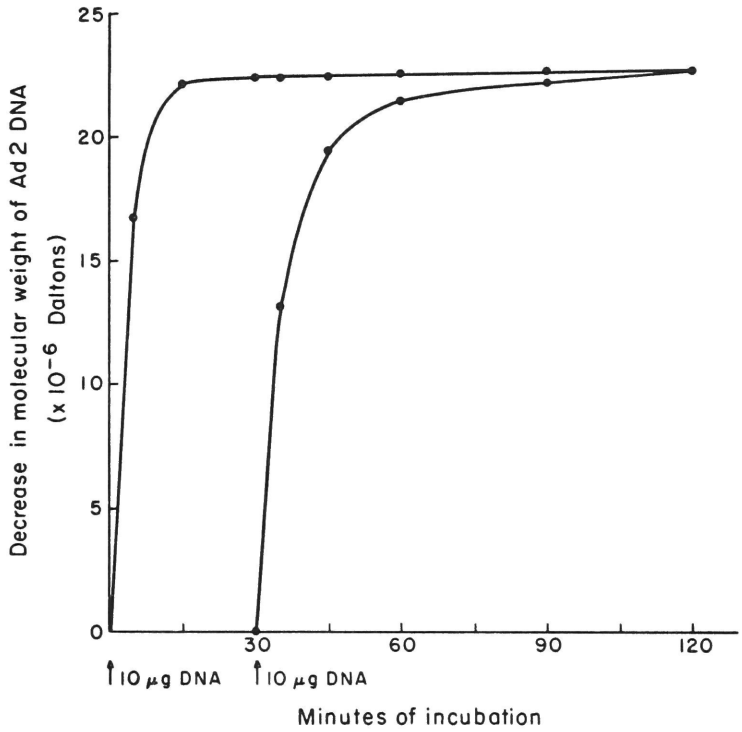

Figure 25: Effect of added substrate on penton endonuclease. The reaction mixture contained 10 μg DNA-^{3}H, 5 μg Ad 2 penton, 100 mM NaCl, 10 mM tris-HCl buffer at pH 7.2, and 2 mM $MgCl_2$. After incubation for 30 min, another sample of 10 μg Ad 2 DNA-^{3}H was added. At intervals, samples were removed, the DNA was extracted and was analyzed in neutral sucrose gradients.

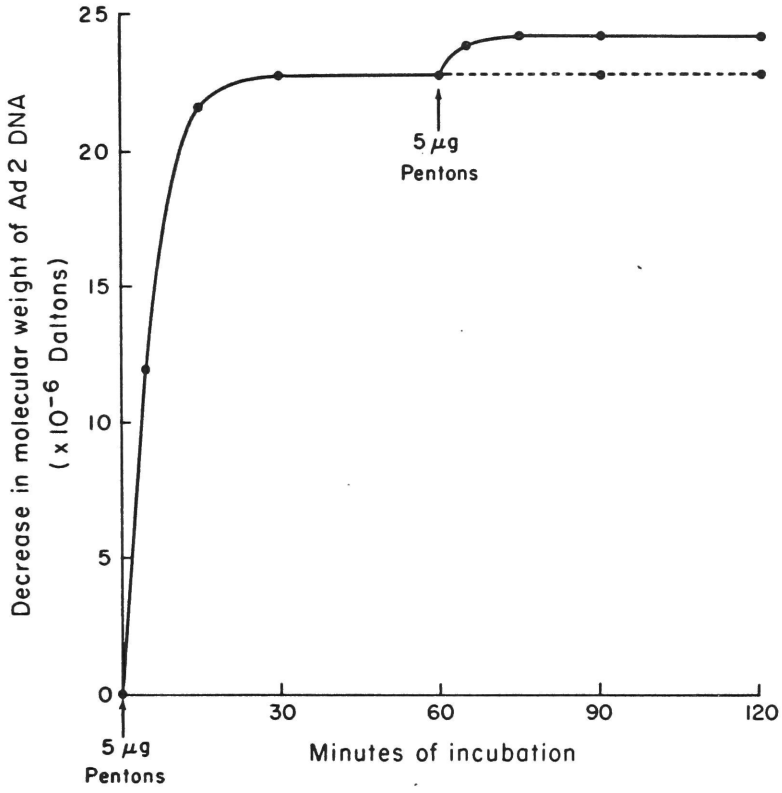

Figure 26: Effect of adding a second sample of penton to the reaction mixture. The reaction mixture contained 10 μg Ad 2 DNA-^{3}H, 5 μg Ad 2 penton, 100 mM NaCl, 10 mM tris-HCl buffer at pH 7.2, and 2 mM $MgCl_2$. After incubation for 60 min, an additional 5 μg of penton was added to the reaction mixture.

immediately cleaved further to a size of 1 million Daltons. It can be concluded that after 60 min of incubation and partial hydrolysis, the adenovirus DNA is still a substrate for the endonuclease.

The results of the last three experiments (a-c) indicate that the DNA and the penton remain reactive throughout the incubation, and that the size of the DNA fragments is not altered further after the initial reaction. The size of the DNA end product is determined by the concentration of the penton. These results suggest that there are specific reaction sites for the penton and that there is an ordered preference for reaction sites on the Ad 2 DNA. When free penton subunits are available, second order sites are attacked only after first order sites have been occupied.

7) Substrate concentration. The effect of substrate concentration on the endonuclease reaction was determined. The data of this experiment are illustrated in Figure 27. When there are less than three penton subunits per Ad 2 DNA molecule, the reaction rate is markedly reduced but the final size of the DNA after 120 min of incubation is nearly constant, regardless of the DNA concentration used. This result again supports the concept of specific binding sites and the requirement of a minimum penton concentration for activity. The results of both this experiment and the previous one imply a low turnover number for the penton subunit.

g. Mechanism of the endonuclease reaction.

1) Characteristics of the DNA product. The DNA product of the penton endonuclease was characterized by zonal sedimentation in both neutral and alkaline sucrose density gradients. The data in Figure 28 illustrate the change in the size of Ad 2 DNA during the reaction with penton.

The Ad 2 DNA substrate sediments as a single species and is distributed symmetrically about the maximum both at pH 7.2 and pH 12.5. After cleavage, the DNA sediments at 20.0 S at pH 7.2 and at 20.7 S at pH 12.5. The molecular weight calculated using the equations of Studier (1965) of the native DNA is 6.1 million Daltons and that of the denatured DNA is

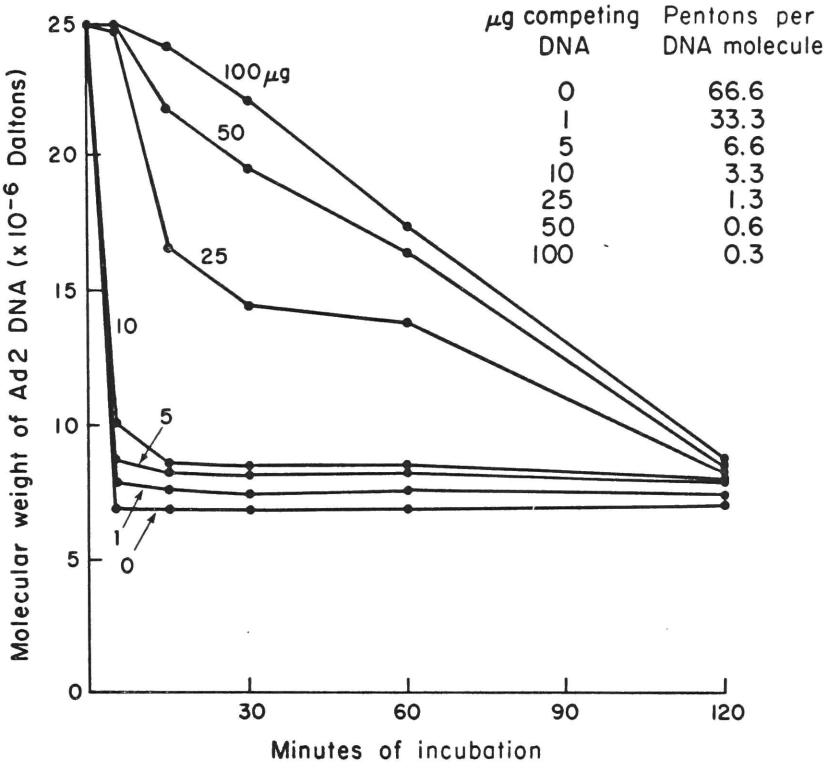

Figure 27: Effect of substrate concentration on the endonuclease reaction. The reaction mixture contained 1 μg of Ad 2 DNA-^{3}H, various amounts of unlabeled Ad 2 DNA, 1 μg of Ad 2 penton, 100 mM NaCl, 10 mM tris-HCl buffer at pH 7.2, and 2 mM $MgCl_2$. At intervals during the incubation, samples were removed, the DNA was extracted and was analyzed in neutral sucrose gradients.

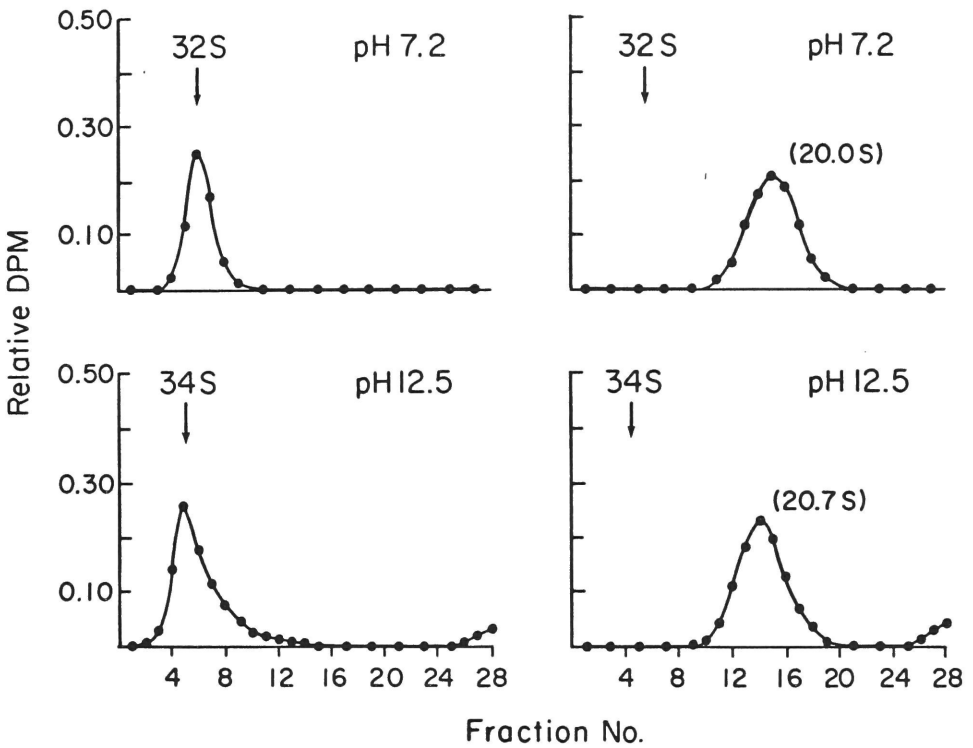

Figure 28: Characteristics of the DNA product from the penton endonuclease reaction. The reaction mixture contained 10 µg Ad 2 DNA-^{3}H, 2.5 µg Ad 2 penton, 100 mM NaCl, 10 mM tris-HCl buffer at pH 7.2, and 2 mM $MgCl_2$. At 0 and 120 min of incubation the DNA was extracted and was analyzed in neutral and alkaline sucrose density gradients.

3.0 million Daltons. There is very little nonsedimenting label found on top of the gradient, indicating that there is little exonuclease activity. Under the conditions described in Figure 28, the Ad 2 DNA is hydrolyzed into fragments, approximately one-fourth the length of the intact DNA. This is similar to the size of Ad 2 fragments found in cells infected with adenovirus. However, the size of the DNA product is related to the concentration of penton used (Fig. 24).

All the experimental evidence available indicate that the size of the reaction product found at pH 7.2 correlates well with that found at pH 12.5 at all time points of the reaction. Thus it can be concluded that the endonuclease makes only double-stranded scissions in the DNA. These scissions do not occur at random since the fragments which are formed have a unique size related directly to the concentration of penton and the type of DNA used. These results indicate that there are specific sites of attack on the DNA molecule. Under these conditions the sites would appear to be the regions rich in guanine and cytosine bases seen on the physical map of the Ad 2 DNA molecule constructed by Doerfler and Kleinschmidt (1970) (see Figs. 2 and 3).

2) Activity of endonuclease during long incubations. All of the data presented indicate that the reaction of the endonuclease with the DNA occurs quickly and goes rapidly to completion with little or no further alteration in the size of the DNA. The data also indicate that there must be more than 0.1 μg of Ad 2 penton/ml to get endonucleolytic cleavage. However, in all of these experiments the size of the DNA was followed during only a 2 hr incubation. At low concentrations, the reaction may occur but be delayed. To test this hypothesis, reaction mixtures were incubated for 48 hr and periodically, DNA samples were removed, extracted and analyzed in alkaline and neutral sucrose gradients.

The two reaction mixtures contained 10 μg Ad 2 DNA-^{3}H, 0.1 or 0.01 μg of penton respectively, 100 mM NaCl, 10 mM tris-HCl buffer at pH 7.2 and 2 mM $MgCl_2$. It can be predicted that these concentrations of penton do not endonucleolytically cleave the Ad 2 DNA during a 2 hr incubation period (see Fig. 27). However, 0.1 μg penton does remove a small amount,

about 1 million Daltons, of the terminal DNA in 2 hr. Even when the reaction was followed for 72 hr, there was no endonucleolytic alteration of the Ad 2 DNA on either alkaline or neutral sucrose density gradients. The removal of the terminal bit of DNA with 0.1 μg of penton did not proceed beyond a 1 million Dalton segment. The segment removed could not be identified in the gradient.

Under the conditions described above, there was 0.5 and 0.05 penton subunits per DNA molecule. When penton-DNA complexes were formed, most of these complexes would have contained only one penton subunit per DNA molecule. Such complexes, if formed, were apparently inactive. These data suggest that more than one penton per DNA molecule is required for activity. The data in Figure 27 suggest that when there are less than 3 penton subunits per DNA molecule, the reaction rate is markedly reduced. These data may suggest that two penton subunits (or more), perhaps one per each strand at a particular site, are required for activity.

Furthermore, the penton-DNA complex once formed must be quite stable since during a long incubation, if there was dissociation of penton-DNA complexes, chance would predict that active complexes would be formed. However, if there was no dissociation, then there would be no activity.

3) Orientation of endonuclease activity to sites with single-strand breaks. The Ad 2 DNA is a linear double-stranded molecule free of single-strand breaks. (Data: Figs. 16, 28; Green et al., 1967b; Doerfler and Kleinschmidt, 1970). Thus the first site of attack of the penton on the native DNA molecule is probably not the site of a pre-existing single-strand scission. However, since only double-strand scissions are found in the end product, an initial single-strand scission may be followed by an immediate second attack which would lead to a double-strand break.

This mode of action was tested by reacting penton with Ad 2 DNA which contained many random single-strand breaks. The substrate sedimented with a symmetrical distribution at 32 S in neutral sucrose gradients. This corresponds to a molecular weight of 24.6 million Daltons. However, when sedimented in alkaline sucrose gradients, a broad sedimentation

pattern was found with a maximum at 9.8 S. This is compatible with a mean molecular weight for the denatured segments of about 0.8 million Daltons.

This DNA (15 µg) was used as a substrate and incubated with 1 µg of penton. During the incubation, samples of DNA were removed, extracted and analyzed in neutral and alkaline sucrose gradients. There was no detectable alteration in the size of the fragments found in alkaline sucrose gradients during the incubation. The neutral sucrose gradients revealed that the DNA was hydrolyzed only at the specific sites which correspond to the regions rich in guanine and cytosine bases. Thus the site of hydrolysis apparently is completely independent of random single-strand breaks.

4) Exonuclease activity. The endonuclease associated with the virus in vivo and in vitro apparently has little exonuclease activity. The penton endonuclease was assayed for exonuclease activity. The penton was mixed with either Ad 2 DNA-^{3}H or Ad 2 DNA-^{14}C in the presence of Mn^{++} or Mg^{++} and incubated. Substrates labeled with different isotopes were used since it was obvious from the physical map (Doerfler and Kleinschmidt, 1970) that the distribution of the bases within the DNA is not homogeneous The ^{3}H is only in thymidine, the ^{14}C is found in both purine bases. The activity of the penton and virion endonuclease were compared. The data of this assay are listed in Table 31.

The endonuclease associated with Ad 2 virions hydrolyzes the Ad 2 DNA to 18.7 S fragments essentially without loss of acid precipitable label. However, the penton endonuclease rapidly hydrolyzes the Ad 2 DNA to smaller sized fragments and much of the label was no longer acid precipitable. The nature of the acid soluble material has not been investigated.

This result has not been found in other assays where the relative penton concentration was lower and has not been found in vivo or in vitro with the virion as a source of endonuclease. This reaction may represent a side reaction or an artifact due to the very high concentration of penton. Since the concentration of penton subunits in the virion and penton assays

Table 31

Products of Virion and Penton Endonuclease Reaction

Length of incubation (min)	Ad 2 virion[a]		Ad 2 vision		Ad 2 penton[b] in Mg^{++} buffer			Ad 2 penton in Mn^{++} buffer		
	S	% acid ppt[c] DNA-^{3}H	S	% acid ppt DNA-^{14}C	S	% acid ppt DNA-^{3}H	% acid ppt DNA-^{14}C	S	% acid ppt DNA-^{3}H	% acid ppt DNA-^{14}C
0	31.0	100	31.0	100	31.0	100	100	31.0	100	100
5	31.0	100	31.0	99.7	24	80.3	81.7	24.4	85.2	76.0
	19.1		20.5							
60	18.8	98.7	18.9	98.0	17	49.4	48.9	11.1	63.2	63.0
120	18.6	96.6	18.6	97.6	17	45.6	45.3	10.8	60.2	53.2

[a] The reaction mixture contained 500 µg of Ad 2 virions and 5 µg Ad 2 DNA in 1 ml of standard buffer.

[b] The reaction mixture contained 10 µg of Ad 2 penton and 5 µg Ad 2 DNA in 1 ml of standard buffer. a and b have comparable amounts of pentons.

[c] Each value for acid precipitable DNA is the mean of 5 determinations.

is the same, this result suggests that either not all of the penton subunits in the virion are active or the associated proteins of the virion regulate the activity of the endonuclease.

5) Substrate specificity. The substrate specificity of the endonuclease was assayed by adding 10 μg of substrate DNA to 1 μg of Ad 2 penton in standard buffer. The initial rate of hydrolysis was determined and the end product was characterized. These results are listed in Table 32.

Nearly any native DNA serves as a substrate for the endonuclease. The rate of the endonuclease reaction with native Ad 2 DNA is at least 20 times greater than with denatured Ad 2 DNA. When a homogeneous species of DNA molecules, like adenovirus or phage λ DNA, are used as a substrate, the end product has a homogeneous size. When cellular DNA, which had been fractionated until it sedimented homogeneously, is hydrolyzed the end product is heterogeneous in size.

This result supports the proposal that the endonuclease has specific sites of attack. The sites of attack on the Ad 2 and Ad 12 molecule apparently are in regions rich in guanine and cytosine bases. When phage λ DNA is hydrolyzed, two homogeneous fragments are formed, one a 27 S fragment and the other a 15 S fragment. The analysis in neutral and alkaline sucrose gradients of a circular λ DNA after the reaction indicates that the λ DNA is hydrolyzed once and opened into a linear molecule.

Neither glucosylated T4 DNA, double-stranded reovirus RNA nor single-stranded cellular RNA will serve as a substrate. Thus the endonuclease is specific for DNA and has a much higher activity with native than denatured DNA.

6) Inhibitors. The penton endonuclease was inhibited with compounds which bind to DNA or which compete with enzymes involved in nucleic acid metabolism. The endonuclease was also treated with physical agents and then assayed for endonuclease activity. The results of these experiments are listed in Table 33.

Table 32

Substrate Specificity of the Penton Endonuclease

Nucleic acid	Estimated relative rate of hydrolysis	Estimated number of cleavages per molecule	Nature of end product
DNA from:			
Ad 2, native	100	3	homogeneous (1)[a]
Ad 2, denatured	5	3	homogeneous (1)
Ad 12, native	100	3	homogeneous (1)
HEK cells	80	many	heterogeneous
KB cells	75	many	heterogeneous
HEL cells	60	many	heterogeneous
BHK cells	80	many	heterogeneous
Phage λ, linear	60	1	homogeneous (2)
Phage λ, circular	40	1	homogeneous (1)
Phage T4	0	none	
RNA from:			
Reovirus	0	none	
KB cells	0	none	
HeLa cells	0	none	

[a] ( ) = number of species found.

Table 33

Inhibition of the Penton Endonuclease

Inhibitor	Concentration	% Inhibition
Competitive:		
tRNA	1 µg/ml	100
	50 ng/ml	55
Actinomycin D	1 µg/ml	32
	10 ng/ml	14
Ethidium bromide	100 ng/ml	8
ATP	10 µM	19
GTP	10 µM	5
dCMP	10 µM	45
$PO_4^{\equiv}$	100 mM	0
Noncompetitive:		
β-mercaptoethanol	10 nM	4
	10 µM	96
EDTA	10 mM	100
SDS	1 %	100
Pyridine	10 %	100
56°C, 30 min		3
Boiling, 10 min		100
Pronase, 30 min	10 µg/ml	100

Only tRNA is a strong and complete inhibitor of the penton endonuclease. E. coli (Lehman et al., 1962) and N. crassa (Linn and Lehman, 1965) endonucleases are also strongly inhibited by tRNA. Dyes like actinomycin D and ethidium bromide which intercalate between the bases of the DNA substrate are weak inhibitors. Eron and McAuslan (1966) noted that exonucleases and endonucleases were inhibited by actinomycin D and ethidium bromide. The inhibition of the endonuclease by actinomycin D indicates that guanine and cytosine base pairs may be involved in the endonuclease reaction. Deoxycytidylic acid is also a moderate inhibitor of the endonuclease. Inhibition by β-mercaptoethanol implies that the endonuclease contains disulfide bonds which are important for activity. However, the amino acid analysis of Ad 2 penton by Pettersson et al. (1969) (see Table 4) indicates that there are no half-cystine residues. Thus the β-mercaptoethanol may act as a reducing agent in the reaction. Physical denaturation or degradation of the endonuclease completely destroys the activity. The endonuclease is stable when heated at 56°C for 30 min but is destroyed when boiled.

7) Effect of synthetic polynucleotide on the endonuclease reaction. Several lines of evidence suggest that the preferred site of attack on the DNA molecule is a region which is rich in guanine and cytosine bases: 1) Hydrolysis which would cleave the specific fragments from Ad 2, Ad 12 and phage λ DNA apparently occur at these sites; 2) the inhibition of the endonuclease by deoxycytodylic acid, and actinomycin D. The hypothesis that this was the preferred site of attack was further tested when dG:dC or dAT was added to the endonuclease reaction. The results of this experiment are shown in Figure 29.

The dAT copolymer is a very poor inhibitor of the endonuclease but the dG:dC is a strong inhibitor. This finding further indicates that the preferred site of hydrolysis of DNA molecules by the Ad 2 endonuclease is a region rich in guanine and cytosine bases.

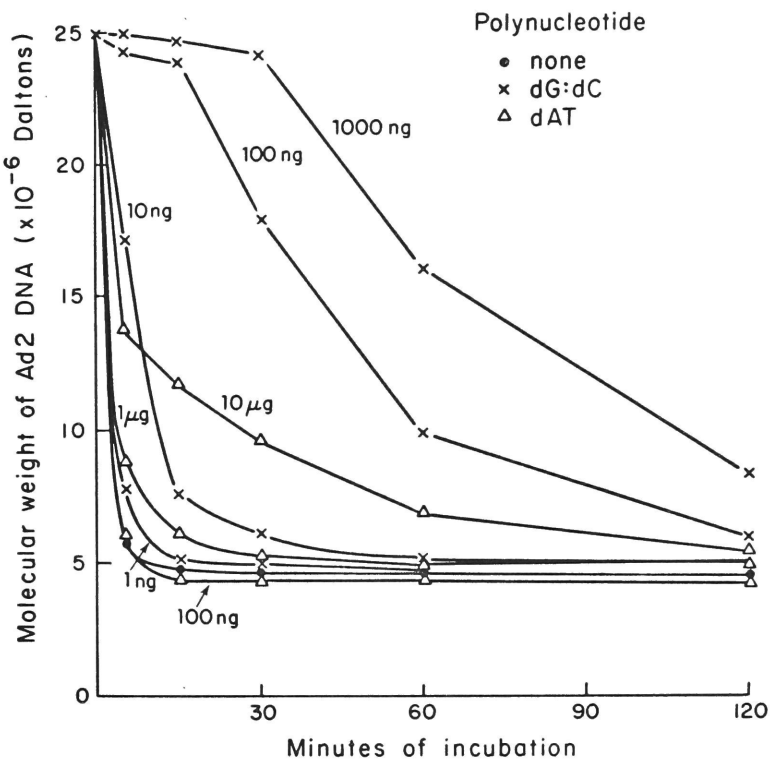

Figure 29: Competition between synthetic polynucleotides and adenovirus type 2 DNA for penton. Each reaction mixture contained various amounts of synthetic polynucleotides, and 1 μg of Ad 2 penton in 1 ml of standard buffer. This mixture was incubated at 0°C for 30 min, then 1 μg of Ad 2 DNA-^{3}H was added and the reaction mixture was incubated at 37°C. Samples were removed at intervals, the DNA was extracted and the size of the DNA was analyzed in neutral sucrose gradients.

C. Summary of Results

1. Presence of the endonuclease

a. In vivo. Cells infected by Ad 2 or Ad 12 with labeled DNA contain three species of labeled viral DNA. One of these species sediments at 18.6 S and is symmetrically distributed. The evidence suggests that this fragment is formed by a preformed endonuclease associated with the virion.

b. In cell extracts in vitro. Extracts of cells infected with Ad 2 and Ad 12 contain the endonuclease activity. The endonuclease activity is found both early and late in productive infection but only early during abortive infection. The early endonuclease activity is not newly synthesized and apparently is derived from the virions which infect the cell. The late endonuclease is regulated or coded by the viral genome and apparently is synthesized at the time capsid proteins are synthesized.

c. In or on the adenovirion in vitro. When purified virions are incubated with adenovirus DNA in vitro, the DNA is cleaved to the same size fragments as found in vivo. Cellular DNA is also hydrolyzed. The DNA within the virion is also hydrolyzed during incubation. The endonuclease activity is specifically inhibited by antiserum and thus the Ad 2 and Ad 12 endonuclease are antigenically distinct. The endonuclease associated with the virion has specific reaction conditions.

d. In the penton base in vitro. When capsid subunits are incubated with adenovirus DNA, only the penton subunits hydrolyze the DNA. The fiber and hexon subunits do not compete with the penton endonuclease for the DNA. The endonuclease activity is specifically inactivated by antiserum or trypsin treatment. The amount of endonuclease activity in partially purified preparations correlates with the amount of penton base antigen present and not with total protein. Thus the endonuclease activity apparently resides in the five, presumably identical polypeptides of the penton base. The reaction conditions are similar to the conditions for the virion associated endonuclease. Thus the endonuclease of the virion is localized in the penton base and this endonuclease is active in vivo.

2. Characteristics of the parental viral DNA in

a. Size, identity and origin. Three species of viral DNA are found in infected cells. The fast sedimenting species (45 S to 80 S) is mainly cellular DNA which contains small amounts of viral DNA, probably in integrated form. This viral DNA may be parental DNA or newly synthesized DNA. The intact viral DNA species sediment (Ad 2, 33 S; Ad 12, 29 S) homogeneously. This DNA may be either parental or newly synthesized and is the predominant form of viral DNA found in the productively infected cells.

The slow sedimenting DNA is symmetrically distributed in alkaline (20.5 S) and neutral (18.6 S) sucrose density gradients. This DNA hybridizes only with viral DNA when the parental DNA is labeled, a small portion (10%) of the newly synthesized viral DNA before 33 hr after infection is the slow sedimenting species of DNA. After this time, the slow sedimenting species of DNA becomes the predominant species of DNA synthesized.

b. Role of these viral DNAs. The intact viral DNA represents the DNA template and the genetic material from the infectious particles. The slow sedimenting DNA apparently is a cleavage product of the intact viral DNA. The fast sedimenting DNA may represent the viral DNA integrated into the cellular genome. The slow sedimenting DNA may be a precursor of the fast sedimenting DNA. The role of the fast sedimenting DNA is not known.

c. Regulation of the species of viral DNA. Each of the viral DNA species appears in regular amounts and at defined times and sequences within the cell, suggesting that these DNA species are regulated by the viral or the cellular genome.

3. Specificity of the endonuclease. The endonuclease is specific for DNA. Native DNA is hydrolyzed at least 20 times faster than denatured DNA. Glucosylated DNA is not hydrolyzed nor is double- or single-stranded RNA. The DNA product appears homogeneous if a homogeneous species of DNA is used as the substrate.

4. Mechanism of action of the endonuclease. The endonuclease apparently makes only specific, double-stranded scissions in DNA molecules. The site of preferred attack apparently is a region rich in guanine and cytosine bases. Some exonuclease activity can be demonstrated in vitro with high concentrations of penton. Exonuclease activity is not associated with the endonuclease in the adenovirions.

5. The endonuclease is a viral gene product. The specific neutralization by specific antiserum indicates that the endonuclease is coded by the viral genome and not the cellular genome. Since the antigenic determinants of the penton base are characteristic for each adenovirus group, this data further indicate that the viral genome codes for the endonuclease. Thus the endonuclease is localized to the polypeptides of the penton base.

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V. GENERAL DISCUSSION

A. Species of Viral DNA Found in Cells Infected by Adenovirus

Both the parental viral DNA and the newly synthesized viral DNA apparently are metabolized similarly. The intracellular forms of parental and newly synthesized viral DNA have the same size and have the same apparent change in size from one species to another. These changes occur at essentially the same time to the parental and newly synthesized DNA. Thus the mechanism by which the parental DNA and the newly synthesized DNA are metabolized is probably the same.

The release of the DNA from the adenovirion in the cell was studied for Ad 5 by Lawrence and Ginsberg (1967) and for Ad 2 by Lonberg-Holm and Philipson (1969). The results of this study agree with their results concerning the release of the parental DNA. The synthesis of the newly formed intact DNA of Ad 2 and Ad 12 is similar to the results reported by Mak (1969).

The fast sedimenting DNA species was not studied thoroughly in these experiments. Clearly viral DNA sediments with the cellular DNA in alkaline sucrose gradients. An equivalent amount of marker DNA added during extraction of the intracellular DNA does not sediment with the cellular DNA. This rules out block sedimentation of the DNA and wall interaction followed by roll-down as a cause for the fast sedimenting DNA. The evidence suggests that the labeled viral DNA is linked with the cellular DNA by covalent bonds. The results presented by Doerfler (1968b) were similar, and in further equilibrium studies the covalent linkage between the labeled DNA and cellular DNA was clearly established.

An alternative hypothesis in the productively infected cells would be that polymeric forms of the viral DNA exist. However, this would require that the parental DNA strands are joined with other parental or newly synthesized viral DNA. These polymers could also arise during synthesis of the new viral DNA and explain the fast sedimenting species. However, these polymeric forms were not isolated, and there is no evidence

for recombination between coinfecting adenoviruses, which may be expected if polymeric forms existed. Also, permutation of the completed genome may be expected from polymeric forms, but the physical map of Ad 2 was unique (Doerfler and Kleinschmidt, 1970). The same amount of fast sedimenting DNA is found in abortively infected cells where no viral DNA is synthesized as in productively infected cells. This eliminates reincorporation of label from the parental DNA in a polymeric form as a source of fast sedimenting viral DNA.

The results in this series of experiments suggest that even during the productive cycle a small amount, 5 to 10%, of the parental DNA, and a few percent of the newly synthesized viral DNA may become covalently linked to the cellular DNA. In this context it is interesting to compare the results presented by zur Hausen (1967, 1968a) and Homma et al. (1968). These authors demonstrated chromosomal breaks in cells productively infected with Ad 2 or Ad 12 and autoradiographic evidence demonstrated that radioactivity from parental virus was frequently associated with these sites. All of these experiments suggest that the association of the adenovirus DNA with the cellular DNA, probably by a covalent linkage, is not an uncommon event or an event unique to the virus transformed cell. Weil (personal communication) similarly found that in SV40 and polyoma infected cells, there was integration of the viral genome in both productive and abortive infections.

The slow sedimenting viral DNA derived from the parental DNA was described by Doerfler (1968a). The characterization of this slow sedimenting species of DNA, the mechanism by which this slow sedimenting species of DNA is synthesized, and its role in the life cycle of the virus formed the central problem of this research.

The DNA-DNA hybridization data clearly indicate that this DNA is derived entirely from viral DNA. In the cell, the slow sedimenting DNA nearly always has a molecular weight of about 5.5 million Daltons and is free of single-strand breaks. The data further suggest that the slow sedimenting DNA is formed from the parental or newly synthesized DNA by

double-strand scissions at specific sites at one-fourth, one-half and three-fourths the length of the intact DNA. This suggests that a specific endonuclease cleaves the slow sedimenting DNA from the intact viral genome.

B. Endonuclease Induced in Cells Infected by Adenovirus

When KB, BHK or HEL cells are infected with a high multiplicity the "early" endonuclease activity is present within the cell from 4 to 8 hr after infection. "Early" endonuclease is the source of the endonuclease activity which appears before viral DNA synthesis starts and corresponds to the time when early enzymes are synthesized. Late endonuclease is the source of the endonuclease activity which appears after viral DNA synthesis starts and corresponds to the time when capsid proteins are being synthesized. When the virus genome is inactivated, only the "early" endonuclease is found within the cells. If the multiplicity of infection is reduced, then the "early" endonuclease declines in correspondence with the multiplicity. The appearance of the "early" endonuclease is not inhibited by chemical inhibitors of macromolecular syntheses. Similar results were described by Pogo and Dales (1969) for vaccinia. Three enzymes were brought into the cell by the vaccinia virion. Although there was no evidence that these enzymes were structural components of the virion, both enzymes were clearly contained within the envelope of the virus.

The "early" endonuclease of the adenovirus, brought in by the infecting virions, probably is responsible for the appearance of the slow sedimenting DNA which first appears at 7 to 8 hr after infection with high multiplicity. It is somewhat surprising that the appearance of the product of the endonuclease is delayed so long in these cells.

The results of Lonberg-Holm and Philipson (1969) indicated that one-half of the virions were uncoated to cores by 30 min. Both Lawrence and Ginsberg (1967) and Lonberg-Holm and Philipson (1969) agreed that only 10% of the viral proteins from the parental virus were hydrolyzed within the cell. The data of Morgan et al. (1969) suggested that none of the parental proteins went into the nucleus with the viral DNA during uncoating.

However, Chardonnet and Dales (1969) stated that only the adenovirions found in the nucleus were replicated. The data of Lonberg-Holm and Philipson (1969) indicated that much of the capsid protein was associated with the partially uncoated virus isolated from infected nuclei. Lonberg-Holm (personal communication) stated that quantitatively about one-half of the capsid proteins (pentons included) were isolated from the nucleus and identified antigenically at 4 hr after infection.

Thus there is ample evidence that the protein of the endonuclease is present at the site of virus uncoating and new viral DNA synthesis, but the endonuclease activity is not detected as early as expected. Thus the endonuclease may be latent in a bound form which is not released until 6 to 7 hr after infection of the cells, or the viral DNA may be protected from the endonuclease. The bound form of endonuclease may be partially uncoated virions or virions within the vacuoles. Protection of the DNA seems a less likely mechanism since Russell et al. (1968) have demonstrated that the viral DNA was sensitive to pancreatic deoxyribonuclease within the capsid once the vertices were removed. Also, the viral endonuclease acts in vitro on the DNA within the virion.

The late endonuclease appears at 14 hr after infection and apparently is newly synthesized and coded for by the viral genome. This is demonstrated by the data obtained from: 1) the experiment with light inactivated virus, and 2) the relative insensitivity to low multiplicity of infection. Thus the late endonuclease is a newly synthesized protein. The time course of endonuclease activity in infected cells suggests that most of the parental viral DNA is cleaved before the late proteins are synthesized. However, the early cleavage of the parental DNA may be an artifact (compared to natural infection) of the high multiplicity of infection. Most of the newly synthesized DNA is not cleaved until 33 hr after infection and later, although a nearly constant 10% of the DNA synthesized at any time is found as slow sedimenting DNA.

The presence of intact newly synthesized viral DNA implies that the DNA is protected from the action of the endonuclease, either by compartmentalization, protein binding, regulation or inactivation of the endonuclease. Late in infection nearly all of the newly synthesized

viral DNA is cleaved, and thus obviously the protective mechanism must no longer be functioning.

It is surprising that extracts of infected cells at 24 hr and later after infection do not have larger amounts of newly synthesized endonuclease activity. There is at least a one-thousandfold increase in the number of virus particles synthesized and they represent only a small portion, about 10%, of the viral protein synthesized. However, these excess proteins probably are sequestered in the inclusion bodies and thus not available. Another alternative is that most of the newly synthesized viral DNA is protected from the endonuclease or the endonuclease is regulated by a cellular or viral mechanism. It is also surprising that with all of this endonuclease activity within the nucleus, not all of the viral DNA is cleaved. Cellular DNA is also cleaved in vitro and in vivo by the endonuclease in these experiments. Such cleavage fragments have been reported by Doerfler (1969) for BHK cells infected with Ad 2 or Ad 12.

C. Endonuclease Associated with the Adenovirion

The fact that the endonuclease of the virion is inhibited by specific antiserum strongly suggests that the endonuclease is coded for by the viral genome. Further, antiserum to Ad 2 penton will specifically inhibit the endonuclease of the Ad 2 virion. This clearly indicates that the endonuclease activity of the virion is due to the penton. This result should be tested further with antisera to other adenoviruses in the group. Pettersson and Höglund (1969) demonstrated that the antigens of the penton base are common among members within the group. The best evidence would be to dissociate the virions and isolate and characterize the protein with endonuclease activity.

The reaction conditions for the penton endonuclease and the virion endonuclease differ mainly in the requirement for divalent ions. The isolated penton subunits require magnesium or manganese ions for activity but the virion endonuclease does not, in fact 0.001 M EDTA does not inhibit the virion endonuclease. Several reasons may be suggested to explain this difference: 1) There are many divalent gegenions associated with the

virion and released during the incubation. The experiment with EDTA suggests that there must be about 10^9 divalent gegenions per virion. These ions could be detected by chemical means; however, this has not been studied. 2) The endonuclease of the virion has divalent ions firmly bound to it during assembly and the isolated penton subunits do not. The in vitro data suggest that 10^6 divalent ions per penton subunit is required for optimal activity. 3) Alternatively, the divalent ions may be bound at the active site on the DNA molecule. Thus they could serve as a component of regulation. If the divalent ion were bound to the DNA, the divalent ion requirement would depend on the DNA concentration. This was not explicitly tested experimentally; however, the reaction kinetics over a 100-fold range of DNA concentration with constant Mg^{++} and penton concentration remained first order with little change in the rate constant. 4) Since only a small portion of the penton subunits which are synthesized are incorporated into virions, there may be a selection for a particular species of penton subunits from a heterogeneous population. Thus the penton subunits isolated from infected cells may have a different specificity than penton subunits derived from the virions. This could be tested by dissociating the virus and purifying the penton fraction. 5) The binding of the DNA by the penton subunit associated with or derived from the virion is different from the binding by isolated penton subunits. The penton subunit released from the vertices of virion is surrounded by 5 hexons in a complete rosette pattern. The isolated penton subunit has none of this associated protein. Levine and Ginsberg (1967a,b) showed that hexons bound to DNA and this binding interfered with DNA- and RNA-polymerase activity. Thus very clearly the associated hexons may influence the activity of the penton subunits.

When the penton concentration is low, the same sized products as are made in vitro by virions and in vivo during infection are formed. However, the penton subunits demonstrate an exonuclease type activity and the size of the DNA end product depends on penton concentration. Exonuclease activity is not found in vivo or in vitro with virions. This

activity may be regulated by associated subunits or their proteins. Since the competition studies did not demonstrate regulation, the regulation must depend on specific interaction between the subunits.

Regulation could also be related to the structure of the viral DNA. Philipson (personal communication) found that Ad 2 DNA prepared by ethanol precipitation was not hydrolyzed by the Ad 2 penton, although the Ad 2 DNA is hydrolyzed if this procedure is not used. He suggested that the resistance of the DNA precipitated with ethanol was due to alteration in the secondary structure of the DNA. Regulation could also be mediated by the histone-like proteins of the adenovirus core or the relationship between the capsid and/or core proteins and the specific sites of attack.

D. The Endonuclease is a Virus-coded Product

The evidence that the endonuclease of Ad 2 and Ad 12 is antigenically unique and specific, clearly suggests that the endonuclease is coded for by two different viral genomes and not by the cell genome. Furthermore, rigorous data establish the purity of the penton preparation; only one intact subunit by electron microscopy, only one species on analytical ultracentrifugation, only one species on polyacrylamide gel electrophoresis at two different pH and only viral protein present on immunoelectrophoresis. The specific inhibition of penton endonuclease by antiserum and further the specific inhibition of Ad 2 virion endonuclease by specific antiserum for Ad 2 penton base. All these data rule out contamination by cellular enzymes and further establish the virus-specific nature of the reaction and endonuclease.

The data on production of the endonuclease in the infected cell clearly establishes that if the viral genome is damaged, there is no synthesis of functional endonuclease. The fact that the endonuclease is synthesized after the virus DNA is synthesized further supports the finding that it is a structural capsid protein. Assay of the capsid subunits clearly establishes that the only subunit with endonuclease activity is the penton. Thus the conclusion that the penton subunit is the endonuclease of the adenovirion is supported by many lines of evidence. The conclusion that the penton base is the endonuclease is well supported

although the isolated penton base cannot be assayed directly for activity. Thus the polypeptides of the penton base act as a structural component of the virion and demonstrate an enzymatic activity in vivo and in vitro.

E. Mechanism of Action and Specificity of the Adenovirus Endonuclease

The DNA product of the endonuclease reaction in vivo and in vitro with extracts of infected cells, purified virions or penton is identical. Also, the reaction conditions are similar. Since the penton is probably the endonuclease in all these reactions, the mechanism of action is probably the same. All of these sources of endonuclease yield a common sized DNA product from Ad 2 and Ad 12 DNA, suggesting that the site of reaction is the same. The native and denatured DNA product sediment as a single species and have corresponding molecular weights. Thus the endonuclease makes only double-stranded scissions in the substrate DNA. This type of mechanism has been classified "double hit" kinetics. Bernardi and Cordonnier (1965) have described three deoxyribonucleases which make double-strand scissions.

The initial hydrolysis is a first order reaction with a rate constant of 0.017 min^{-1}. The activation energy for the reaction is 12,000 calories/mol. The endonuclease is heat stable. The endonuclease is inhibited by β-mercaptoethanol although the enzyme apparently contains no cystine on amino acid analysis (Pettersson et al., 1969). The endonuclease activity is completely inhibited by salt concentrations above 0.5 M. Any native DNA apparently will serve as a substrate. The reaction rate with native DNA is 20 times the reaction rate with denatured DNA. Glucosylated DNA, double-stranded RNA and single-stranded RNA do not serve as a substrate for the endonuclease.

The end product is homogeneous when a homogeneous species of DNA is used as a substrate. The end product is heterogeneous when a uniform-sized cellular DNA is used as a substrate. Furthermore, when the fragments of DNA are compared with the physical maps of the homogeneous species of DNA, the preferred sites of attack apparently are regions rich in guanine and cytosine bases. Polydeoxyguanylic acid-polydeoxycytodylic acid is a

very strong inhibitor of the reaction while the alternating copolymer of deoxyadenylic acid and deoxythymidylic acid is a poor inhibitor. These data support the hypothesis that the preferred site of hydrolysis is at regions rich in guanine and cytosine bases.

F. Role of the Adenovirus Endonuclease

1. Cytopathic effect. Pereira (1960) identified the cytotoxic factor of five different adenoviruses as the penton subunits. The cells were killed by the noninfectious cytotoxic factor within 24 hr. Levine and Ginsberg (1967a,b) reported that the fiber adsorbed to viral and cellular DNA in vitro and inhibited both DNA- and RNA-polymerase by competition. Bello and Ginsberg (1967) demonstrated that synthesis of host cell enzymes is markedly inhibited at the time penton subunits are synthesized. Ledinko (1966, 1968) clearly showed that there is no exonuclease activity associated with Ad 2 and Ad 12 virions in vitro nor an increase in exonuclease in cells productively or abortively infected by Ad 2 or Ad 12. The in vitro experiment described in this report and Doerfler (1969) indicate that large amounts of cellular DNA are hydrolyzed to 8 to 14 S fragments when penton subunits are synthesized.

Thus the mechanism of the cytotoxic effect may be two-fold. The fiber of the penton competes with the DNA- and RNA-polymerase, halting replication and transcription. The penton base may hydrolyze the template DNA. Thus the DNA being transcribed would be susceptible to inhibition by the fiber and double-stranded scissions by the penton base. This would explain the irreversible generalized decline in cell protein and DNA synthesis which occurs only after viral DNA is synthesized (Bello and Ginsberg, 1967). And this inhibition could be the early event which eventually leads to the death of the cell. The penton endonuclease could also play a role in causing the relatively specific chromosomal breaks described in cells infected with adenovirus by Cooper et al. (1967), Stich and Yahn (1967), zur Hausen (1967, 1968a) and Homma et al. (1968).

It is not clear why the viral DNA is not likewise destroyed by the penton. Clearly, the work of Hayashi and Russell (1968) and Velicer and Ginsberg (1970) indicates that soon after DNA synthesis starts, there is

an abundance of penton in the nucleus. The data indicate that at the time the cellular DNA is cleaved, only about 10% of the viral DNA is cleaved. Early during infection, the viral DNA may be specifically protected from the effect of the endonuclease by some mechanism. However, late in the productive infection, after 33 to 35 hr, nearly all of the newly synthesized viral DNA is hydrolyzed.

Thus the endonuclease may contribute to the cytotoxic effect of the penton subunit by cleaving the cellular DNA which is transcribed. The endonuclease also may play a key role in the formation of chromosomal breaks associated with adenovirus infection.

2. Integration. The data in this series of experiments suggests that the slow sedimenting viral DNA formed from the parental or newly synthesized viral DNA by the penton endonuclease is a precursor for the fast sedimenting DNA found in the cells. The integration of viral DNA occurs rather specifically at the time viral DNA synthesis starts in productively infected cells. Weil (personal communication) finds that the DNA of polyoma and SV40 is also integrated during productive infection.

Since the endonuclease may have a relative specificity based on clusters of specific base pairs in both the viral and cellular DNA, the terminal nucleotides of the cellular and viral DNA fragments would potentially have a great deal of homology. If a limited amount of hydrolysis by an enzyme with specificity similar to exonuclease III from E. coli (Richardson et al., 1964) removed a few terminal bases from one strand, then the cellular and viral DNA could potentially form a hydrogen bonded strand. This hydrogen bonded strand containing both cellular and viral DNA could then be covalently linked by cellular repair enzymes or a specific ligase. The release of acid soluble fragments by the high concentrations of penton or the penton endonuclease activity in the presence of manganese ions suggests the penton may provide the terminal exonuclease III type activity also.

The correlation between guanine plus cytosine content and oncogenicity noted by Green et al. (1967a) may be partially explained by the apparent preference of the endonuclease for guanine and cytosine rich sites.

The data of Gilead and Ginsberg (1966), Freeman (1967a,b), and Rainbow and Mak (1970) indicated that a specific and relatively large segment of viral DNA was required for transformation; from one-half to one-fourth of the viral genome is required for transformation. The endonuclease could specifically form this fragment or a larger fragment. The lower the guanine plus cytosine content of the adenovirus DNA, the more likely a large fragment would be formed. This fragment again would potentially have a high degree of homology with the cellular DNA at the site of the endonucleoytic cleavage. However, there is no evidence that integration of the viral DNA into the cell is an essential step in transformation of cells by adenovirus.

3. Defective genomes. The adenoviruses have an unusually high ratio of physical particles to infectious particles. The DNA and proteins from all of the particles apparently are identical and all of the particles are uncoated, but only one particle in three hundred to ten thousand may replicate. The reason for this is unknown. In a series of experiments not discussed here, Ad 2 and Ad 12 particles were preincubated in buffer and then cells were infected with these particles. The DNA within these particles was fragmented, the portion of fragmented DNA depended on the temperature and length of incubation. However, the infectivity titer was considerably greater than the number of particles with intact genomes. Lonberg-Holm and Philipson (personal communication) find that nearly all of the adenovirions adsorbed to the cell contain fragmented DNA but after uncoating, the viral DNA is intact. Rainbow and Mak (1970) report that most of the noninfectious particles express some viral functions in cells but most particles do not express enough functions to produce infectious virus.

The penton endonuclease may play a role in the production of these defective particles and defective infections. The restoration of full infectivity to some of the particles implies the genome is repaired. This implies a specific scission is made in the DNA without loss of any genetic material and the scission is then repaired by cellular or viral enzymes.

4. Messenger template. The endonuclease may hydrolyze the large viral genome into smaller fragments which serve as templates for messenger RNA synthesis. This may enhance regulation since some entire segments could be repressed by viral core or structural proteins. The genes should be clustered into functional groups if this were true. If the DNA segments from the endonuclease reaction were isolated and were hybridized with mRNA isolated from abortively infected cells and from productively infected cells at early and late times after infection, this hypothesis could be tested.

Weil (personal communication) suggested that the DNA of polyoma and SV40 must be "physically associated" (integrated) with the cellular DNA even in the productively infected cells for transcription. This may also be true for adenoviruses. In each cell line infected with either type of adenovirus, about 5 to 10% of the parental DNA sedimented with the cellular DNA at times later than 10 to 12 hr after infection. This is the time when new DNA synthesis starts. This may suggest that during adenovirus infection, the DNA for late protein synthesis must be integrated before transcription occurs. Thus, late mRNA should hybridize better with fast sedimenting viral DNA than with early mRNA.

5. Recombination. The endonuclease could be instrumental for recombination. Thus far, preliminary experiments using mixed infection with Ad 2 and Ad 12 have not demonstrated any recombination. Mak (1969) has reported similar results. However, such experiments should be performed with mutants of the same type of adenovirus, but good adenovirus mutants are not available yet.

There is ready recombination between SV40 DNA and adenovirus DNA and hybrids encapsidated in adenovirus capsids are formed (Baum et al., 1966; Levin et al., 1969). The amount of SV40 genome varies but the total length of the DNA in the capsid is about that expected for the original adenovirus DNA. The endonuclease may make the specific scission so specific segments of adenovirus and SV40 DNA may be joined.

6. Scission of polymeric viral DNA. If the adenovirus genome is synthesized as a polymeric form, then the endonuclease could specifically cleave these polymers into intact genomes. When the newly synthesized DNA was analyzed in neutral and alkaline sucrose gradients, 4 to 50% of the newly synthesized viral DNA sediments as a size predicted for dimeric and trimeric adenovirus genomes. This DNA hybridizes only to viral DNA. However, if polymers were synthesized, and there are three equally preferred sites of cleavage, there should be permutation of the adenovirus genome; but the physical map of Ad 2 is unique (Doerfler and Kleinschmidt, 1970).

However, the polymeric form could contain highly preferred sites at the termini of the genome which are removed entirely. Thus permutation may not be seen, especially at low endonuclease concentration. The incomplete cleavage of the terminal regions may explain why some of the Ad 2 DNA molecules have a short region which is rich in guanine and cytosine bases at the terminus and not present on other molecules (see Fig. 2). This would also explain the release of a small amount of label from the termini when the viral DNA is incubated with very small amounts of endonuclease. The adenovirus DNA is synthesized several hours before the new endonuclease is synthesized, thus there should be an accumulation of polymeric forms; however, this is not seen. When the rate of cleavage in vitro is extrapolated to the conditions within the nucleus, the endonuclease from the parental virion should be adequate to cleave all of the viral DNA synthesized.

The endonuclease may function in the formation of adenovirus-SV40 hybrids by specifically cleaving the recombinants into segments of an appropriate length which may be encapsidated into adenovirus capsids.

7. Source of DNA for incomplete particles. The DNA isolated from the incomplete adenovirus particles is the same size as the end product of the endonuclease reaction. This suggests that the cleaved DNA is specifically or fortuitously incorporated into these particles.

VI. ADDENDUM

VI. ADDENDUM

The endonuclease activity contained in cells infected with adenovirus types 2 or 12 is not present in mock-infected cells. This endonuclease activity hydrolyzes the viral and cellular DNA, both the parental and the newly-synthesized DNA, at specific times during the growth cycle. The endonuclease apparently is a viral protein because: 1) Cells in which macromolecular syntheses has been chemically inhibited contain endonuclease activity 4 to 7 hr after infection. 2) Cells infected with inactivated adenovirus contain endonuclease activity which is apparently brought into the cell by the virion, but the endonuclease activity does not increase at 12 to 24 hr after infection as it does in cells infected with control virus. 3) The amount of the endonuclease activity at 4 to 7 hr after infection is directly related to the multiplicity of infection but the endonuclease activity at 12 to 24 hr is not related to multiplicity of infection if the multiplicity is greater than one. The endonuclease activity has not been associated with a protein isolated and purified from extracts of cells infected with adenovirus. The endonuclease activity in infected cells has not been identified with viral proteins by using specific antiserum or polyacrylamide gel electrophoresis with viral protein as marker.

The preformed nature of the endonuclease is confirmed by the in vitro experiments which use the purified virion as a source of endonuclease. Other cellular enzyme activities found in extracts of cells infected with adenovirus; protease, exonuclease and ATPase, are not associated with the purified virion. The endonuclease activity of Ad 2 and Ad 12 grown in KB cells is specifically inhibited by antiserum. This suggests that the endonuclease is virus coded or there is one cellular endonuclease activated or induced upon infection but the antigenicity is specifically modified by the adenovirus which infects the cell or there are two different cellular endonucleases which are specifically induced by the adenovirus and adsorbed to the virion or penton subunit. Although a soluble endonuclease activity is released simultaneously with the penton and hexon subunits when the adenovirion is heat inactivated, purified virions have not been dissociated

and the endonuclease isolated. The subunit or protein with endonuclease activity should be isolated and purified from the virion, and then identified in polyacrylamide gels. Also mutants of adenovirus which do not have endonuclease activity should be sought and the alteration in protein structure identified. Both of these experiments would confirm the data which indicate the endonuclease is a structural component of the virion. However, good methods which dissociate the subunits or peptides from the virion have not been described. Preliminary experiments with "salting-in" techniques suggest that the virions are dissociated and the peptides can be separated. End group analysis of the active protein may help identify contaminants. Since all adenovirus capsid peptides have alanine as the N-terminal residue, another N-terminal residue would indicate contamination.

Only the penton subunit has endonuclease activity. This endonuclease activity is specifically inhibited by antiserum. The endonuclease activity associated with the virion and the penton have similar requirements for optimal reaction conditions and form similar end products. The data suggest that the penton base contains the endonuclease but penton bases with activity have not been isolated. The fiber may maintain a specific active configuration of the peptides in the penton base. The peptides of the penton base have not been dissociated and assayed for endonuclease activity. Isolated peptides from the penton base could be purified much further than the subunits and adsorbed proteins which may contaminate the penton may be eliminated.

Contamination by cell proteins has been evaluated by physical techniques. The virion preparations are 99.99% pure and the penton preparations are at least 99.9% pure. Although further resolution may be attained by analysis of N-terminal residues, contamination of the penton preparation cannot be rigorously ruled out. Two results which could support the existence of a contaminant are the biphasic pH optima and the exonuclease activity present when highly concentrated penton preparations were assayed. Since some enzymes have multiple pH optima, the biphasic optimum does not necessarily mean a second enzyme is present. The biphasic optimum could also be explained by the binding of penton subunits by either penton base

or fiber. This explanation assumes that the maxima of the pH curve represent an optimal pH for the binding of one of the penton components to DNA and that the endonuclease is active whether the penton is bound by the fiber or the base. The exonuclease activity is found only when the penton concentration is very high. Thus the exonuclease activity could represent a very small-sized product formed by high activity of the endonuclease or a contamination by an exonuclease. The bulk of the data indicate that the endonuclease is a structural component of the virion and not a contaminant. Analysis of the peptides present in the preparation will help resolve this problem.

The mechanism of the endonuclease must be studied further. The dissociation of the penton subunit into peptides will determine if the intact subunit is required or if each of the peptides of the penton base is an active molecule of endonuclease. The cleavage reaction is presumably a hydrolysis although phosphorolysis has not been ruled out. This can be determined by using isotopically labeled water or phosphate in the reaction and identifying the location of the label in the end products. Since the DNA substrate has only one count per minute per molecule, the specific activity of the substrate must be increased before exonuclease activity by the penton or virion can be strictly ruled out. This will probably require DNA labeled with phosphate-^{32}P and a complete analysis of the small molecules liberated during the reaction. The endonuclease activity should be assayed by the number of end groups present as well as the size of the DNA present. This can be done by using the polynucleotide kinase reaction with γ-AT^{32}P. This assay will identify the number of terminii on the DNA and the presence of a terminal phosphate and enable identification of the terminal bases. This will also help confirm the site of specificity. The specificity of the reaction can be checked further by using synthetic polynucleotides with known sequences as substrate.

VII. APPENDICES

VII. Appendices

Appendix A: Media and Solutions Used

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APPENDIX A: MEDIA AND SOLUTIONS USED

1. Media and solutions used for culturing cells

a. Media. The ingredients for the media used for cell cultures are listed in Table A1. The ingredients in each section were prepared as concentrated stock solutions, stored frozen, and when required were thawed, diluted and mixed in the order listed and sterilized by filtration. The sterility for mycoplasma and bacteria by culture was checked and the toxicity for HeLa cells was determined. The media were stable for several weeks when stored at 4°C.

b. Tryptose phosphate broth. Tryptose phosphate broth was used as a protein supplement for cell cultures. It was also used experimentally as a replacement for serum or as a supplement with low concentrations of serum. These experiments indicated cells could be maintained in media without serum but they would not divide. The ingredients of tryptose phosphate broth are listed in Table A2. The results of an analysis of tryptose are described in Table A3. The solution was sterilized by filtration. The sterility for bacteria was checked by culture. This solution does not contain antibiotics and occasionally was responsible for contamination of cell cultures.

c. Phosphate buffered saline. Phosphate buffered saline (PBS) was originally described by Dulbecco and Vogt (1954). The composition of PBS is listed in Table A4. Usually PBS is sterilized by filtration since antibiotics are present. It can be autoclaved only without antibiotics. This PBS was frequently used as a washing solution for cells and was used as a reaction buffer for many experiments.

d. Trypsin-versene solution. The cells were suspended from monolayers with trypsin-versene solution. The composition of the trypsin-versene solution is listed in Table A5; the solution was sterilized by filtration.

2. M-9 medium for culturing bacteria. E. coli for bacteriophage production were grown in M-9 medium described by Adams (1959). The composition of M-9 medium is listed in Table A6.

3. Pfannstil agar medium for culturing mycoplasma. The preparation of Pfannstil agar is listed in Table A7. The Pfannstil agar was melted, 2.5 ml horse serum and 20,000 units of penicillin were added to each tube and the medium was poured into petri plates for use.

Table A1

Ingredients of Media for Cell Culture

Media	Eagle's[a]	Spinner[b]	Reinforced Eagle's[c]	HEK[d]
Salt Solution:				
NaCl	6.8 g	6.8 g	6.8 g	16.5 g
KCl	0.4	0.4	0.4	1.0
$NaH_2PO_2 \cdot H_2O$	0.15	1.5	0.15	0.34
Na_2HPO_4	-	-	-	-
$Na_2HPO_4 \cdot 7H_2O$	-	-	-	-
$MgCl_2 \cdot 6H_2O$	0.2	0.2	0.2	-
$MgSO_4 7H_2O$	-	-	-	0.50
KH_2PO_4	-	-	-	
$CaCl_2$	0.2	-	0.2	0.50
Dextrose	1.0	1.0	4.5	2.4
Phenol red	0.016	0.016	0.016	0.039
Amino Acids II:				
L-cystine	24.0 mg	24.0 mg	48.0 mg	137.3 mg
L-tryosine	36.0	36.0	72.0	205.9
Amino Acids I:				
L-arginine	105.0	105.0	210.0	675.6
L-histidine	31.0	31.0	62.0	177.3
L-isoleucine	52.0	52.0	104.0	297.4
L-leucine	52.0	52.0	104.0	297.4
L-lysine	58.0	58.0	116.0	331.8
L-methionine	15.0	15.0	30.0	85.8
L-phenylalanine	32.0	32.0	64.0	183.0
L-threonine	48.0	48.0	96.0	274.6
L-tryptophan	10.0	10.0	20.0	57.2
L-valine	46.0	46.0	92.0	263.1

Table A1 (Cont.)

Non-essential amino acids				
L-alanine		8.9 mg		127.3 mg
L-asparagine		15.0		214.5
L-aspartic acid		13.3		190.2
L-glutamic acid		14.7		210.2
L-glycine		7.5		107.3
L-proline		11.5		164.5
L-serine		10.5		150.2
L-glutamine	292.0	292.0	584.0	832.2

Vitamins:	Eagle's	Spinner	Reinforced Eagle's	HEK
Choline	1.0	1.0	4.0	2.8
Pyridoxal	1.0	1.0	4.0	2.9
Riboflavin	0.1	0.1	0.4	0.3
Thiamine	1.0	1.0	4.0	2.9
Inositol	2.0	2.0	8.0	5.8
Nicotinamide	1.0	1.0	4.0	2.9
Sodium panthothenate	1.0	1.0	4.0	2.9
Folic acid	1.0	1.0	4.0	2.9
Sodium bicarbonate	2.0 gm	2.0 gm	3.7 gm	11.78 gm
Sodium pyruvate	-	-	.11	
Ferric nitrate	-	-	0.1 mg	
NaOH				684.8 mg

Antibiotics:				
Penicillin-G	500,000 units	500,000 units	500,000 units	1,430,000 unit
Streptomycin	100 mg	100 mg	100 mg	286 mg
Mycostatin	25,000 units	25,000 units	25,000 units	
Fungizone				14.3 mg

The components are listed in order in which they are added to the medium.

[a] Eagle (1955).

[b] Eagle (1959).

[c] Bablanian et al. (1965).

[d] Strohl et al. (1967).

Table A2

Composition of Tryptose Phosphate Broth

Ingredient	Grams per liter
NaCl	5.0
$Na_2HPO_4 \cdot 7H_2O$	2.0
Dextrose	2.0
Tryptose (Difco)	20.0

Table A3

Composition of Tryptose[a]

Ash	8.5%
Total nitrogen	14.0%
Amino nitrogen	5.0%

Amino acids	Percent of amino acid
Arginine	5
Aspartic acid	7
Glutamic acid	15
Glycine	7
Histidine	2
Isoleucine	4
Leucine	6
Lysine	7
Methionine	2
Phenylalanine	4
Threonine	3
Tyrosine	5
Valine	5

Vitamin factors	µg/gm
Pyridoxine	2.8
Biotin	0.4
Thiamine	1.7
Nicotinic acid	71.0
Riboflavin	5.6

[a] Wheat (1970).

Table A4

Composition of Phosphate Buffered Saline[a]

Ingredient	Quantity per liter
NaCl	8.00 g
KCl	0.20
$Na_2HPO_4 \cdot 7H_2O$	1.15
$MgCl_2 \cdot 6H_2O$	0.10
KH_2PO_4	0.20
$CaCl_2$	0.10
Penicillin-G	500,000 units
Streptomycin	100 mg
Mycostatin	25,000 units

[a] Dulbecco and Vogt (1954).

Table A5

Composition of Trypsin-Versene Solution

Ingredient	Quantity per liter
NaCl	8.0 g
KCl	0.20
Na_2HPO_4	1.15
KH_2PO_4	0.20
Trypsin	5.0
EDTA	1.0
Streptomycin	0.100
Penicillin	500,000 units

Table A6

Composition of M-9 Medium[a]

Ingredient	Quantity per liter
Na_2HPO_4	7.00 g
KH_2PO_4	3.0
NaCl	0.5
NH_4Cl	1.0
Glucose	4.0
$CaCl_2$	0.011
$MgSO_4$	0.12
Gelatin	0.010
Casamino acids	10.0
Glycine	3.4 ml

[a] Adams (1959).

Table A7

Preparation of Pfannstil Agar[a]

Pfannstil agar:

a. 2.5 1 beef heart infusion
b. 37.5 g Difco agar
c. heat to melt agar
d. filter through three layers of cotton
e. distribute into tubes (15 ml)
f. autoclave for 15 min at 121° C

Beef heart infusion:

a. 10 lb beef heart (fat removed) and 10.5 1 H_2O
b. make infusion at 80° C
c. store in cold overnight
d. filter out beef
e. 10 1 filtered infusion
   + 100 g peptone
   + 50 g NaCl
   + about 240 ml 1N NaOH (to pH 8.0)
f. boil for 15 minutes

[a] Nelson (1954).

APPENDIX B: SUPPLEMENTARY INFORMATION FOR DETERMINATION OF RADIOACTIVITY

1. Analysis of double-isotope counting methods. Okita et al. (1957) evaluated the accuracy of three methods of determining the radioactivity of two isotopes in the same sample. The screening method had a standard error of 7.6% for ^{3}H and 6.7% for ^{14}C. The simultaneous equation method had a standard error of 4.7% for ^{3}H and 8.4% for ^{14}C. The discriminator ratio method had a standard error of 3.7% for ^{3}H and 4.6% for ^{14}C. Thus the discriminator ratio method clearly has the greatest accuracy.

The amplification and window settings of the liquid scintillation spectrometer were adjusted until samples with oxygen quenching but no self-absorption had less than 4% of the ^{3}H spectrum overlapping the ^{14}C spectrum and less than 10% of the ^{14}C spectrum overlapping the ^{3}H spectrum. The counting efficiency for ^{14}C was 75.90% and for H was 28.81%. Using these settings, the constants for the discriminator ratio calculations are determined for each type of sample: aqueous, neutral sucrose, alkaline sucrose, neutral CsCl, alkaline CsCl, in Bray's scintillation fluid or for desiccated samples in Toluene-Liquifluor(R). These constants are accurate (less than 5% standard error) for ^{3}H to ^{14}C ratios from 0.25 to 50. Thus the amount of marker-^{14}C added to samples is carefully adjusted to be one-fifth the number of counts per minute of the ^{3}H sample.

2. Counting characteristics of fluor-sample. Alkaline and neutral sucrose gradients were collected into vials and a 10 µl sample of DNA with labeled ^{3}H or ^{14}C was added to alternate vials. Scintillation fluid was added and the radioactivity in the sample was determined at least six times. The data were analyzed for the self-absorption gradient due to the solute of gradient by the Scintillation Parameter Program described below. The self-absorption gradient is linear when related to the fraction number. The data for self-absorption of the DNA label by the solute are found in Table A8.

Table A8

Parameters Defining Self-Absorption of Radioactivity in Sucrose Gradients as a Function of Fraction Number[a]

	Neutral sucrose gradients		Alkaline sucrose gradients	
Parameter	Slope	Intercept	Slope	Intercept
External standard ratio	-.0002	.6959	-.0001	.7450
A[b]	-.003	.0402	.0002	.0483
B[c]	.0065	3.2587	.0395	2.4463
^{3}H-CPM/sample	15.4200	1066.03	176.17	11,873.7
^{14}C-CPM/sample	1.2759	243.05	11.35	1,142.1

[a] Approximately 0.200 ml fractions of 5 to 20% sucrose gradients were collected in vials. Alternate functions received 10 µl of ^{3}H or ^{14}C labeled DNA. Each fraction was counted in 10 ml of Bray's scintillation fluid.

[b] ^{3}H CPM in channel 2/^{3}H CPM in channel 1.

[c] ^{14}C CPM in channel 2/^{14}C CPM in channel 1.

Theoretically, if sample quenching is important, each of the parameters should be linearly correlated with the external standard ratio. However, there is no correlation between the parameters and the external standard ratio. Thus the linear correlation with the fraction number is interpreted to mean that quenching does not vary for the samples but slef-absorption due to the solute was the basis for the variation. Since this correction is linearly related to the fraction number, these correction factors are utilized to calculate the data from gradients. Bray's liquid scintillation fluid is far superior to other scintillation fluids and solubilizers checked for aqueous samples.

3. Scintillation Parameter Program. The data determining the parameters of the scintillation system are calculated using the Scintillation Parameter Program listed as Program 1. All programs are written in modified Fortran I for the Control Data Corporation, model 160 G digital computer. Most programs are stored in a program library in compiled form and can be called by a Fortran Bootstrap, thus eliminating the 10 to 15 min compilation time for each program.

The Scintillation Parameter Program allows 100 samples, each with the four variables: external standard ratio (ESR), counts per minute in channels one (C1), two (C2), and three (C3). These data must have been previously processed by Data Check and Data Correct Programs described in Appendix D. These two programs correct the errors made by the automatic card punch attached to the liquid scintillation counter and store the corrected data on magnetic tape.

There are three optional branches in the program. Sense switch one, when on, halts the program after each data unit is calculated and is plotted so the color of the plotter pen may be changed. If sense switch two is on, data for all three channels is analyzed, but if off, only the data for channels one and two is analyzed. If sense switch four is on, ^{3}H are the even numbered samples and ^{14}C are the odd. If sense switch four is off, ^{14}C are the even numbered samples and ^{3}H are the odd numbered samples.

158844

The program must be supplied with one control card and one constant card. The control card contains the number of the tape and the file where the data are stored and the number of gradients to be processed (Format 20). The constant card contains the background for channels one, two and three in counts per minute (Format 2).

The data is then retrieved from the data storage tape, the background is subtracted, and the A and B terms calculated. All data A, B, ^{3}H and ^{14}C are plotted as a function of fraction number and external standard ratio. The external standard ratio is also plotted as a function of fraction number. For each plot, the axis intervals are automatically calculated from the range of data points. The slope and intercept of the data is calculated using a least squares curve fit to a second order equation. The simultaneous equations were solved by the method of Gauss elimination with no row interchanges required. The slope and intercept for each plot are stored as a three dimensional matrix.

When the plotting for all nine or eleven graphs is done for the data of a gradient, the pen returns to the origin of the first graph and the program is reinitiated for the next set of data from the gradient. Thus each set of data is plotted on the same graph and there is one line and set of data points for each. Each line on the graph is identified by the number of the set of data. When all sets of data for the gradient are calculated and plotted, the slope, intercept and mean value for each set of data is printed. Then the means for these three values are calculated and printed for each parameter.

COMPUTER PROGRAM 1

```
*     SCINTILLATION PARAMETERS- BYRON BURLINGHAM- 502 FOUNDERS- 6 NOV.
      DIMENSION ESR(100), C1(100), C2(100), C3(100), CC1(100), CC2(100),
     1CC3(100), A(100), B(100), X(100), Y(100), DX(10),
     2 DY(10), DATA(11,20,3),  VAR1(20), VAR2(20), VAR3(20), BAD(2,3),
     3Z(10), YO(11), YSC(11), SD(11), YMEAN(11), XO(11), XSC(11)
      EQUIVALENCE (C1, CC1), (C2, CC2), (C3, CC3), (A, B ),
     1           (C1, VAR1), (C2, VAR2), (C3, VAR3)
 2000 FORMAT (1H1, 20X, 10A6, 20X, I5, I5)
 2001 FORMAT (1H0, 10X, 3HAES, 20X, 3HAER, 20X, 2HAA)
 2002 FORMAT (10X, 3HAIS, 20X, 3HAIR, 20X, 2HAA)
 2003  FORMAT (10X, 3HEIS, 20X, 3HEIR, 20X, 2HAE)
 2004 FORMAT (10X, 3HBES, 20X, 3HBER, 20X, 2HAB)
 2005  FORMAT ( 10X, 3HBIS, 20X, 3HBIR, 20X, 2HAB)
 2006 FORMAT (10X, 3HCIS, 20X, 3HCIR, 20X, 2HAC)
 2007 FORMAT (10X, 3HCES, 20X, 3HCER, 20X, 2HAC)
 2008 FORMAT (10X, 3HHIS, 20X, 3HHIR, 20X, 2HAH)
 2009 FORMAT (10X, 3HHES, 20X, 3HHER, 20X, 2HAH)
 2010 FORMAT (10X, 3HXES, 20X, 3HXER, 20X, 2HAX)
 2011 FORMAT (10X, 3HXIS, 20X, 3HXIR, 20X, 2HAX)
C     SENSE SWITCH 2-  3 CHANNEL ANALYSIS IS ON
C     SENSE SWITCH 1--  IF ON- STOPS AFTER EACH DATA UNIT
C     IF SENSE SWITCH 2, N IS SPECIAL, NOT GENERAL
C     H3= EVEN, C14= ODD-- SENSE  SWITCH 4 OFF, VICE VERSA-  ON
      I= MDUMPF (3)
    1 PAUSE 4
      REWIND 7
      READ 20, NUMB, NNUMB, IK
   20 FORMAT (I5, I2, I5)
      IZ= IK
      IF (NUMB) 94, 94, 95
   95 I= XFILEF (7, NUMB)
   94 READ 2, BH, BC, BX
    2 FORMAT (3F10.5)
      K=1
    3 IF (SENSE SWITCH 1) 300, 400
  300 PAUSE 2
  400 READ INPUT TAPE 7, 40, (Z(I), I= 1,10), N
   40 FORMAT (10A6, I3, 8X)
      READ INPUT TAPE 7, 4, (ESR(I), C1(I), C2(I), C3(I), I= 1, N)
    4 FORMAT (4F12.4)
      DO 5,I= 1, N
      CC1(I)= C1(I) - BH
      CC3(I)= C3(I) - BX
    5 CC2(I)= C2(I) - BC
      J= 2
      M= 1
      IF (SENSE SWITCH 4) 301, 302
  301 J= 1
      M= 2
  302 DO 6, I= J, N,  2
    6 A(I)= CC2(I)/CC1(I)
      DO 7, I= M, N,  2
    7 B(I)= CC2(I)/CC1(I)
      KZ= 9
      KZA= 9
      IF (SENSE  SWITCH 2) 71, 100
   71 KZ= 11
      KZA= 11
      N= N - 11
      GO TO 700
   10 DO 10000, I= 1,2
      DO 10000, JJ= 1,3
```

```
10000 BAD(I, JJ)= 0.0
      DO 11, I= M, N, J
      BAD(1,1)= BAD(1,1) + 1.0
      BAD(1,2)= BAD( 1,2) + X(I)
      BAD(1,3)= BAD(1,3) + Y(I)
      BAD(2,1) = BAD(1,2)
      BAD(2,2)= BAD(2,2) + X(I)**2
   11 BAD(2,3)= BAD(2,3) + (X(I)*Y(I))
      DO 21720, I= 1,2
      SAD= BAD(I,I)
      DO 21721, JJ= 1,3
21721 BAD(I,JJ)=BAD(I, JJ)/SAD
      DO 21720,JJ= 1,2
      IF(JJ-I) 21722, 21720, 21722
21722 CAD= BAD(JJ, I)
      DO 21720, L= 1,3
      BAD(JJ,L)=BAD(JJ,L)- BAD(I,L)*CAD
21720 CONTINUE
      S= BAD(2,3)
      R= BAD(1,3)
      DO 21, I= M, N, J
      Q= PLOTF ( X(I), Y(I), 3)
      PRINT (PLOTTA)221, IZ
  221 FORMAT (3H  1, I2)
   21 CONTINUE
      DO 13,  I= 1, 9
      SDEV= (I - 1)* 1.000
      DX(I)= XO(KZ) + XSC(KZ) * SDEV
   13 DY(I)= S*DX(I) + R
      Q=PLOTF (DX(1), DY(1), 3)
      TOP = YO(KZ) + YSC(KZ)*10.0
      DO 14, I=  1,9
      IF ( DY(I) - TOP)  140, 140, 14
  140 IF (DY(I) - YO(KZ)) 14, 141, 141
  141 Q=PLOTF(DX(I), DY(I), 4)
   14 CONTINUE
      PRINT ( PLOTTA)221, IZ
      Q= PLOTF (XO(KZ), (YO(KZ) + 15.0*YSC(KZ)),3 )
      DATA(KZ, IZ, 1) = S
      DATA(KZ, IZ, 2) = R
      DATA(KZ, IZ, 3) = YMEAN(KZ)
      KZ= KZ - 1
  700 IF(KZ- 11) 701, 702, 100
  702 DO 7020, I= 1, N
      X(I)= ESR(I)
7020 Y(I)= CC3(I)
      IF (K) 7021, 7021, 7022
7022 CALL SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      CALL RANGE(X, XO, XSC, N, KZ)
      YO(KZ)= YMEAN(KZ) - SD(KZ)*4.30
      YSC(KZ)= SD(KZ)*1.2000
7021 CALL PLOT (XO, XSC, YO, YSC, KZ, K )
      GO TO 10
  701  IF( K7- 10) 100, 703, 100
  703 DO 7030, I= 1, N
7030 X(I)= I
      IF (K) 7031, 7031, 7032
7032 CALL SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      XO(KZ)= 0.000
      NSC = N/9.4
      XSC(KZ)= NSC + 1.00
7031 CALL PLOT (XO, XSC, YO, YSC, KZ, K )
```

```
      GO TO 10
  100 IF (KZ - 9) 103, 102, 200
  102  M= 2
      J= 2
      IF (SENSE SWITCH 4) 1023, 1024
 1023 M= 1
 1024 DO 101,  I= 1, N
      Y(I)= A(I)
  101 X(I)= ESR(I)
      CALL SIGMA (Y, M,    J, SD, YMEAN, KZ, N)
      IF (K) 1021, 1021, 1022
 1022 YO(KZ)= YMEAN(KZ) - SD(KZ)*4.300
      YSC(KZ)= SD(KZ)*1.2000
      CALL RANGE (X, XO, XSC, N, KZ)
 1021 CALL PLOT (XO, XSC, YO, YSC, KZ, K )
 1051 GO TO 10
  103 IF (KZ - 8) 106, 105, 200
  105 DO 1054, I= 1, N
 1054 X(I)= I
      CALL SIGMA (Y, M,    J, SD, YMEAN, KZ, N)
      IF (K) 10541, 10541, 10542
10542 YO(KZ)= YO(9)
      YSC(KZ)= YSC(9)
      XO(KZ) = 0.00
      NSC = N/9.4
      XSC(KZ)= NSC + 1.00
10541 CALL PLOT (XO, XSC, YO, YSC, KZ, K )
 1057 GO TO 10
  106 IF (KZ - 7) 109, 108, 200
  108 DO 1084, I= 1, N
 1084 Y(I)= ESR(I)
      CALL SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      M= 1
      J= 1
      IF (K) 10841, 10841, 10842
10842 YO(KZ) = YMEAN(KZ) - SD(KZ)*4.00
      YSC(KZ)= SD(KZ)* 0.800
      XO(KZ)= XO(8)
      XSC(KZ)= XSC(8 )
10841 CALL PLOT (XO, XSC, YO, YSC, KZ, K )
10821 GO TO 10
  109 IF (KZ - 6) 112, 111, 200
  111 J= 2
      IF (SENSE SWITCH 4) 1116, 1117
 1116  M= 2
 1117 DO 1110, I= M, N, 2
      X(I) = ESR(I)
 1110 Y(I) = B(I)
      CALL SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      IF (K) 1111, 1111, 1112
 1112 YO(KZ)= YMEAN(KZ) - SD(KZ)*4.00
      YSC(KZ)= SD(KZ)*0.800
      XO(KZ)= XO(9)
      XSC(KZ)= XSC(9)
 1111 CALL PLOT (XO, XSC, YO, YSC, KZ, K )
 1114 GO TO 10
  112 IF(KZ -5) 114, 115, 200
  115 DO 1150 I= M,  N, 2
 1150  X(I)= I
      CALL SIGMA (Y, M,    J, SD, YMEAN, KZ, N)
      IF (K) 1153, 1153, 1154
 1154 XO(KZ)= 0.00
```

```
      XSC(KZ)= 4.000
      YO(KZ) = YO(6)
      YSC(KZ)= YSC(6)
1153  CALL PLOT (XO, XSC, YO, YSC, KZ, K )
1151  GO TO 10
 114  IF(KZ- 4) 116, 117, 200
 117  IF (SENSE SWITCH 4) 1174, 1181
1174  M= 2
1181  DO 1170 I= M,  N, 2
1170  Y(I) = CC2(I)
1175  CALL SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      IF (K)    1171, 1171, 1172
1172  XO(KZ) = 0.000
      XSC(KZ) = 4.0000
      YSC(KZ)= SD(KZ)*0.800
      YO(KZ)= YMEAN(KZ) - SD(KZ)*4.00
1171  CALL PLOT (XO, XSC, YO, YSC, KZ, K )
1173  GO TO 10
 116  IF(KZ-3) 118, 119, 200
 119  DO 1190, I= M, N, 2
1190  X(I) = ESR(I)
      CALL SIGMA (Y, M,    J, SD, YMEAN, KZ, N)
      IF (K) 1193, 1193, 1194
1194  XO(KZ)= XO(9)
      XSC(KZ)= XSC(9)
      YO(KZ) = YO(4)
      YSC(KZ)= YSC(4)
1193  CALL PLOT (XO, XSC, YO, YSC, KZ, K )
1191  GO TO 10
 118  IF( KZ - 2) 120, 121, 200
 121  M= 2
      IF (SENSE SWITCH 4) 1212, 1215
1212  M= 1
1215  DO 1210, I= M, N, 2
      X(I)= I
1210  Y(I)= CC1(I)
      CALL SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      IF (K) 1213, 1213, 1214
1214  XO(KZ)= 0.000
      XSC(KZ) = 4.000
      YO(KZ)= YMEAN(KZ) - SD(KZ)*4.00
      YSC(KZ)= SD(KZ)*0.800
1213  CALL PLOT (XO, XSC, YO, YSC, KZ, K )
1211  GO TO 10
 120  IF (KZ - 1) 200, 123, 200
 123  DO 1230, I= M, N, 2
1230  X(I) = ESR(I)
      CALL SIGMA (Y, M,    J, SD, YMEAN, KZ, N)
      IF (K) 1234, 1234, 1235
1235  XO(KZ)= XO(9)
      XSC(KZ)= XSC(9)
      YO(KZ)= YO(2)
      YSC(KZ) = YSC(2)
1234  CALL PLOT (XO, XSC, YO, YSC, KZ, K )
1231  GO TO 10
 200  Q=PLOTF( XO(1), YO(1), 3)
      DO 2001, KZ= 2, KZA
      Q=PLOTF (XSC(KZ), YSC(KZ), 1)
      Q=PLOTF (XO(KZ), YO(KZ), 2)
      Q=PLOTF (XO(KZ), YO(KZ), 3)
2001  Q=PLOTF (XO(KZ), (YO(KZ) - 15.0*YSC(KZ)), 3)
      K=K-1
```

```
      IZ = IZ - 1
      IF (IZ) 202, 202, 3
  202 PRINT 2000, (Z(I), I= 1, 10), NNUMB, NUMB
      DO 211, K= 1, KZA
      GO TO (31, 32, 33, 34, 35, 36, 37, 38, 39, 40, 41) K
   31 PRINT 2009
      GO TO 42
   32 PRINT 2008
      GO TO 42
   33 PRINT 2007
      GO TO 42
   34 PRINT 2006
      GO TO 42
   35 PRINT 2005
      GO TO 42
   36 PRINT 2004
      GO TO 42
   37 PRINT 2003
      GO TO 42
   38 PRINT 2002
      GO TO 42
   39 PRINT 2001
      GO TO 42
   40 PRINT 2011
      GO TO 42
   41 PRINT 2010
   42  DO 212, I= 1, IK
       VAR1(I) = DATA(K, I, 1)
       VAR2(I) = DATA(K, I, 2)
  212  VAR3(I) = DATA(K, I, 3)
      OS= 0.
      OR = 0.
      OA = 0.
      DO 203, I= 1, IK
      OS= OS + VAR1(I)
      OR = OR + VAR2(I)
  203 OA = OA + VAR3(I)
      OSA= OS / IK*1.0
      ORA= OR / IK*1.00
      OAA = OA / IK*1.00
      PRINT 2100,(VAR1(I), VAR2(I), VAR3(I), I= 1,IK)
 2100 FORMAT (5X, F14.4, 10X, F14.4, 10X, F14.4)
      PRINT 2200,OSA, ORA, OAA
 2200 FORMAT(1H0, 5X, F14.4, 10X, F14.4, 10X, F14.4, //)
  211 CONTINUE
      REWIND 7
      END
      SUBROUTINE RANGE (X, XO, XSC, N, KZ)
      DIMENSION X(100), XO(11), XSC(11)
      DO 10221, I= 1, N
      J= 1
10230 IF (I - J ) 10229, 10223, 10229
10229 AMAX= X(I) - X(J)
      IF(AMAX) 10221, 10223, 10223
10223 J= J + 1
      IF (J - N) 10230, 10230, 10224
10221 CONTINUE
10224  XO(KZ) = X(I) + X(I)*0.20
      DO 10225, I= 1, N
      J= 1
10226 IF (I - J) 10231, 10227, 10231
10231 AMIN = X(I) - X(J)
```

```
      IF(AMIN) 10227, 10227, 10225
10227  J= J + 1
      IF (J - N) 10226, 10226, 10228
10225 CONTINUE
10228 AMIN= X(I)
      XSC(KZ)= (XO(KZ)-AMIN)/ 5.000
      XO(KZ)=AMIN - XSC(KZ)
      RETURN
      END
      SUBROUTINE SIGMA (Y, M, J, SD, YMEAN, KZ, N)
      DIMENSION Y(100), YMEAN(11), SD(11)
      KOUNT= 0
      SDEV = 0.0
      SUMY= 0.0
      DO 22, I= M, N, J
      SUMY= SUMY + Y(I)
   22 KOUNT= KOUNT + 1
      YMEAN(KZ)= SUMY / KOUNT*1.000
      DO 23, I= M, N, J
   23 SDEV=((YMEAN(KZ) - Y(I))**2.000) + SDEV
      SD(KZ)= SQRTF(SDEV/KOUNT*1.000)
      RETURN
      END
      SUBROUTINE PLOT (XO, XSC, YO, YSC, KZ, K)
      DIMENSION XO(11), XSC(11), YO(11), YSC(11)
      Q=PLOTF (XSC(KZ), YSC(KZ), 1)
      Q=PLOTF (XO(KZ), YO(KZ), 2)
      Q=PLOTF (XO(KZ), YO(KZ), 3)
      IF (K) 1057, 1057, 1056
 1056 Q= PLOTF (XO(KZ), YO(KZ), 3)
      DO 10526, I= 1, 10
      T= YO(KZ) + YSC(KZ)*I
      Q=PLOTF (XO(KZ), T, 4)
      Q= PLOTF ((XO(KZ) -XSC(KZ)*0.20), T, 4)
      Q=PLOTF  ((XO(KZ) -XSC(KZ)*0.20), T, 3)
      Q=PLOTF  ((XO(KZ) -XSC(KZ)*1.00), T, 3)
      PRINT (PLOTTA) 10524, T
10524 FORMAT (3H  1,F10.4)
      Q=PLOTF  ((XO(KZ) -XSC(KZ)*0.20), T, 3)
10526 Q=PLOTF (XO(KZ), T, 4)
      Q=PLOTF (XO(KZ), YO(KZ), 4)
      Q=PLOTF (XO(KZ), YO(KZ), 3)
      Q= PLOTF ((XO(KZ) - XSC(KZ)*1.25),(YO(KZ)+YSC(KZ)*4.0),3)
      GO TO (1,1, 3, 3, 5, 5, 7, 8, 8, 10, 10) KZ
    1 PRINT (PLOTTA) 1001
 1001 FORMAT (3H  1, 2HH3)
      GO TO 20
    3 PRINT (PLOTTA) 1003
 1003 FORMAT  (3H  1, 3HC14)
      GO TO 20
    5 PRINT (PLOTTA) 1005
 1005 FORMAT (3H  1, 1HB)
      GO TO 20
    7 PRINT (PLOTTA) 1007
 1007 FORMAT (3H  1, 3HESR)
      GO TO 20
    8 PRINT (PLOTTA) 1008
 1008 FORMAT ( 3H  1,  1HA)
      GO TO 20
   10 PRINT (PLOTTA) 1010
 1010 FORMAT (3H  1, 2HC3)
      GO TO 20
```

```
   11 PRINT (PLOTTA) 3001
 3001 FORMAT (3H  1, 15HFRACTION NUMBER)
      GO TO 1057
   12 PRINT (PLOTTA) 3002
 3002 FORMAT (3H  1, 3HESR)
      GO TO 1057
   20 Q=PLOTF (XO(KZ), YO(KZ),3)
      DO 10521, I= 1, 10
      T= XO(KZ) + XSC(KZ)*(I-1)
      Q=PLOTF (T, YO(KZ), 4)
      Q=PLOTF ( T, (YO(KZ) - YSC(KZ)*0.20), 4)
      Q=PLOTF ( T, (YO(KZ) - YSC(KZ)*0.20), 3)
      Q=PLOTF ((T-0.5*XSC(KZ)), (YO(KZ)-0.40*YSC(KZ)), 3)
      PRINT (PLOTTA) 10522, T
10522 FORMAT (3H  1,F 8.4)
      Q=PLOTF ( T, (YO(KZ) - YSC(KZ)*0.20), 3)
10521 Q=PLOTF (T, YO(KZ), 4)
      Q=PLOTF (XO(KZ), YO(KZ), 4)
      Q=PLOTF (XO(KZ), YO(KZ), 3)
      Q=PLOTF ((XO(KZ) + XSC(KZ)*4.0), (YO(KZ)- YSC(KZ)*0.75), 3)
      GO TO (12, 11, 12, 11, 11, 12, 11, 11, 12, 11, 12) KZ
 1057 RETURN
      END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
I	4	2 6434	NOT USED
I	3	2 6444	2 4356
KOUNT	3	2 6456	NOT USED
J	2	2 6474	2 4372
I	2	2 6476	2 4400
NSC	1	2 6611	NOT USED
L	1	2 6656	2 4414
JJ	1	2 6674	2 4424
KZA	1	2 6676	NOT USED
KZ	1	2 6702	2 4436
M	1	2 6704	NOT USED
J	1	2 6706	NOT USED
N	1	2 6723	NOT USED
K	1	2 6725	2 4446
IZ	1	2 6746	2 4454
IK	1	2 6756	NOT USED
NNUMB	1	2 6760	NOT USED
NUMB	1	2 6762	NOT USED
I	1	2 6764	2 4462
IO	0	3 7775	2 4504

FLOATING POINT VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION
T	4	2 6431
Q	4	2 6436
SUMY	3	2 6446
SDEV	3	2 6451
AMIN	2	2 6463
AMAX	2	2 6471
OAA	1	2 6550
ORA	1	2 6553
OSA	1	2 6556

OA	1	2	6561
OR	1	2	6564
OS	1	2	6567
TOP	1	2	6631
SDEV	1	2	6634
Q	1	2	6645
R	1	2	6650
S	1	2	6653
CAD	1	2	6660
SAD	1	2	6663
BX	1	2	6733
BC	1	2	6736
BH	1	2	6741

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT	CODE LOCATION	DIMENSION	DIM1	DIM2
XSC	1	2	7315	1		
XO	1	2	7356	1		
YMEAN	1	2	7417	1		

SD	1	2 7460	1		
YSC	1	2 7521	1		
YO	1	2 7562	1		
Z	1	2 7623	1		
BAD	1	2 7661	2	2	
DATA	1	2 7703	3	11	20
DY	1	3 3577	1		
DX	1	3 3635	1		
Y	1	3 3673	1		
X	1	3 4347	1		
ESR	1	3 5023	1		
VAR3	1	3 6153	1		
VAR2	1	3 6627	1		
VAR1	1	3 7303	1		
B	1	3 5477	1		
A	1	3 5477	1		
CC3	1	3 6153	1		
C3	1	3 6153	1		
CC2	1	3 6627	1		
C2	1	3 6627	1		
CC1	1	3 7303	1		
C1	1	3 7303	1		

CONSTANTS

VALUE	OBJECT CODE LOCATION
0.75000000 E 00	2 6301
0.40000000 E 00	2 6313
0.50000000 E 00	2 6316
0.12500000 E 01	2 6417
0.20000000 E 01	2 6441
0	2 6454
0.50000000 E 01	2 6460
0.20000000 E 00	2 6466
5	2 6572
6	2 6574
0.80000000 E 00	2 6576
0.40000000 E 01	2 6601
8	2 6604
0.94000000 E 01	2 6606
0.12000000 E 01	2 6613
0.43000000 E 01	2 6616
0.15000000 E 02	2 6621
4	2 6624
0.10000000 E 02	2 6626
0.10000000 E 01	2 6666
0.0	2 6671
9	2 6700
7	2 6744
2	3 7757
3	3 7761
20	3 7763
11	3 7765
10	3 7767
100	3 7771
1	3 7773

SUBPROGRAMS

IDENT	LEVEL	OBJECT CODE LOCATION

PLOT	4	2 3065
RANGE	2	2 2372
SIGMA	3	2 2671

INTEGER VARIABLES USED AS SUBPROGRAM ARGUMENTS

IDENT	LEVEL	ERASABLE LOCATION	UP SUBROUTINE
K	4	0 0006	NOT USED
KZ	4	0 0005	2 4350
N	3	0 0007	NOT USED
K7	3	0 0006	2 4364
J	3	0 0003	NOT USED
M	3	0 0002	NOT USED
KZ	2	0 0005	2 4406
N	2	0 0004	NOT USED

FLOATING POINT ARRAYS USED AS SUBPROGRAM ARGUMENTS

IDENT	LEVEL	ERASABLE LOCATION	DIMENSION	DIM1	DIM2
YSC	4	0 0004	1		
YO	4	0 0003	1		
XSC	4	0 0002	1		
XO	4	0 0001	1		
YMEAN	3	0 0005	1		
SD	3	0 0004	1		
Y	3	0 0001	1		
XSC	2	0 0003	1		
XO	2	0 0002	1		
X	2	0 0001	1		

STATEMENT NUMBERS

IDENT	LEVEL	OBJECT CODE LOCATION
10521	4	2 4060
12	4	2 3623
11	4	2 3615
20	4	2 3631
10	4	2 3607
8	4	2 3601
7	4	2 3573
5	4	2 3565
3	4	2 3557
1	4	2 3551
10526	4	2 3377
1056	4	2 3157
1057	4	2 4232
23	3	2 3014
22	3	2 2744
10228	2	2 2633
10227	2	2 2603
10231	2	2 2560
10226	2	2 2547
10225	2	2 2623
10224	2	2 2505
10223	2	2 2455
10229	2	2 2432
10230	2	2 2421
10221	2	2 2475
203	1	2 2221
212	1	2 2127

42	1	2 2071
41	1	2 2065
40	1	2 2057
39	1	2 2051
38	1	2 2043
37	1	2 2035
36	1	2 2027
35	1	2 2021
34	1	2 2013
33	1	2 2005
32	1	2 1777
31	1	2 1771
211	1	2 2354
202	1	2 1660
2001	1	2 1577
1231	1	2 1471
1235	1	2 1402
1234	1	2 1432
1230	1	2 1301
123	1	2 1261
1211	1	2 1246
1214	1	2 1151
1213	1	2 1207
1210	1	2 1050
1215	1	2 1022
1212	1	2 1014
121	1	2 1002
120	1	2 1250
1191	1	2 0767
1194	1	2 0700
1193	1	2 0730
1190	1	2 0577
119	1	2 0557
118	1	2 0771
1173	1	2 0544
1172	1	2 0447
1171	1	2 0505
1175	1	2 0364
1170	1	2 0346
1181	1	2 0326
1174	1	2 0320
117	1	2 0314
116	1	2 0546
1151	1	2 0301
1154	1	2 0212
1153	1	2 0242
1150	1	2 0111
115	1	2 0071
114	1	2 0303
1114	1	2 0056
1112	1	1 7760
1111	1	2 0017
1110	1	1 7657
1117	1	1 7631
1116	1	1 7623
111	1	1 7611
112	1	2 0060
10821	1	1 7576
10842	1	1 7501
10841	1	1 7537

1084	1	1	7364
108	1	1	7346
109	1	1	7600
1057	1	1	7333
10542	1	1	7232
10541	1	1	7274
1054	1	1	7131
105	1	1	7113
106	1	1	7335
1051	1	1	7100
1022	1	1	6763
1021	1	1	7041
101	1	1	6662
1024	1	1	6636
1023	1	1	6630
200	1	2	1473
102	1	1	6610
103	1	1	7102
7032	1	1	6434
7031	1	1	6536
7030	1	1	6407
703	1	1	6371
7022	1	1	6165
7021	1	1	6317
7020	1	1	6140
702	1	1	6114
701	1	1	6360
141	1	1	5771
140	1	1	5754
14	1	1	6007
13	1	1	5653
21	1	1	5600
21722	1	1	5434
21721	1	1	5365
21720	1	1	5474
11	1	1	5301
10000	1	1	5166
10	1	1	5132
700	1	1	6103
100	1	1	6577
71	1	1	5104
7	1	1	5044
6	1	1	5004
302	1	1	4764
301	1	1	4750
5	1	1	4710
400	1	1	4536
300	1	1	4535
3	1	1	4531
95	1	1	4476
94	1	1	4511
1	1	1	4434
0	1	1	4425

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION	
10522	4	2	6304
3002	4	2	6321
3001	4	2	6330

1010	4	2 6346
1008	4	2 6355
1007	4	2 6364
1005	4	2 6373
1003	4	2 6401
1001	4	2 6410
10524	4	2 6422
2200	1	2 6500
2100	1	2 6526
221	1	2 6637
4	1	2 6710
40	1	2 6714
2	1	2 6727
20	1	2 6750
2011	1	2 6766
2010	1	2 7010
2009	1	2 7032
2008	1	2 7054
2007	1	2 7076
2006	1	2 7120
2005	1	2 7142
2004	1	2 7164
2003	1	2 7206
2002	1	2 7230
2001	1	2 7252
2000	1	2 7276

LIBRARY FUNCTIONS

IDENT	LEVEL	OBJECT CODE LOCATION
(16120		0 7264
(R405I		0 7340
(R162I		0 7424
PLOTTA		1 0001
INPUT		1 1725
A**B		1 2411
A**I		1 2455
EXPF		1 2545
LOGF		1 2765
SQRTF		1 3143
MDUMPF		1 3273
PLOTF		1 4041
XFILEF		1 4345

ERASABLE STORAGE 2 4516 TO 2 6301

APPENDIX C: SUPPLEMENTARY INFORMATION FOR EQUILIBRIUM SEDIMENTATION

1. Chemical analysis of cesium chloride. The ultrapure cesium chloride supplied by the Harshaw Chemical Company was analyzed for trace contaminants. The absorption at 260 nm of a 50% solution is 0.02 ODU for a 1 cm light path. The trace metals in the CsCl are listed in Table A9. Weygand et al. (1951) have reported that $Pb(OH)_2$ hydrolyzes DNA. Helleiner and Butler (1955) have reported that $Ba(OH)_2$ hydrolyzes DNA. However, the ion concentrations were 0.02 to 0.30 M and the temperature was 100°C. Thus the trace amounts of lead and barium at lower temperatures probably could not hydrolyze the DNA. The suppliers of the other reagents claim that the heavy metal ions are low, less than 5 parts per million. However, complete trace metal analysis was not available. Control DNA incubated at 37°C for 4 hr in the reagents is not hydrolyzed.

2. Accuracy of buoyant density determinations. The accuracy of the buoyant density determinations in the analytical ultracentrifuge was evalauted. Stock suspensions of adenovirus proteins, incomplete virions and complete virions were made in CsCl solution. Samples of the stock suspensions were taken and the refractive index and buoyant density of each suspension were determined five times. These data are found in Table A10. The standard deviation in all cases is less than 0.001 g/cm^3 and in all cases, except with complete virions, a difference of 0.001 g/cm^3 would be highly significant.

3. Density of bacteriophage f1. Bacteriophage f1 was used as a reference marker for the buoyant density determinations. The buoyant density of f1 is similar to phage fd (Zinder, personal communication). Knippers and Hoffman-Berling (1966) reported the buoyant density of phage fd from preparative gradients to be 1.285 g/cm^3. Since phage f1 is used as a reference, the buoyant density must be determined directly. Phage f1 was used as a reference marker over a wide range of density for virions, incomplete virions and proteins. Thus the effect of pressure on the buoyant density of phage f1 was an important factor.

Table A9

Chemical Analysis for Contaminants in Cesium Chloride[a]

Element	maximum concentration (parts per million)
Lead	0.01
Copper	0.01
Nickel	0.005
Cobalt	0.005
Iron	0.01
Zinc	0.01
Aluminum	0.01
Manganese	0.01
Thallium	0.01
Magnesium	0.05
Calcium	0.05
Strontium	5.00
Barium	5.00
Lithium	0.40
Sodium	5.00
Potassium	5.00
Rubidium	10.00

[a] E. Merck A. G., Kontroll-Laboratorium, 61 Darmstadt, Frankfurter Strausse 250, West Germany.

Table A10

Accuracy of Buoyant Density Determinations
in the Analytical Ultracentrifuge

Sample	Mean buoyant density	Standard deviation	Standard error of the mean
Ad 2 virion proteins	1.27681	.000128	.000286
Ad 2 incomplete virions			
Ia	1.29065	< .000001	.000447
Ib	1.29108	.000296	.000663
II	1.29660	.000114	.000255
Ad 2 complete virions	1.32737	.000972	.002174

Solutions of CsCl with various average densities were prepared and the buoyant density of f1 was determined three times for each solution. The average density of the samples was determined by pycnometry. The buoyant density corrected for activity was plotted vs the distance of the phage band from the meniscus and the regression line was determined. These data are seen in Figure A1. When the regression line is extrapolated to the meniscus, the pressure on the phage f1 is 0.0 atm. Thus the buoyant density of 1.3163 g/cm^3 should represent the absolute density of phage f1.

Since the buoyant density of phage f1 is a function of the pressure or distance from the meniscus, the density of the phage f1 reference is calculated from the equation of the regression line in Figure A1:

$$\rho_{f1} = 1.3163 - 0.00364\ (r_{f1} - r_m)_{mm}$$

r_m = radius of meniscus in mm

r_{f1} = radius of phage f1 band in mm

4. Equilibrium sedimentation program. The method of calculating buoyant density described by Ifft et al. (1961) was translated into a computer program and used for the calculation of the buoyant density. The Density Equilibrium Sedimentation Program is printed as Program 2. There are three options in this program. If select switch one is on, the corrected density of phage f1 is used as the reference. If sense switch one is off, the density listed on the second constant card is used as the reference. If sense switch two is on, the refractive index of the solution on the data card is used to calculate the density of the solution. ($\rho_{25} = 10.8601 \cdot \eta_{25} - 13.4974$, Ifft et al., 1961.) If sense switch two is off, the density on the data card was calculated by pycnometry. If sense switch four is on, the reference density is the average density of the solution in the cell.

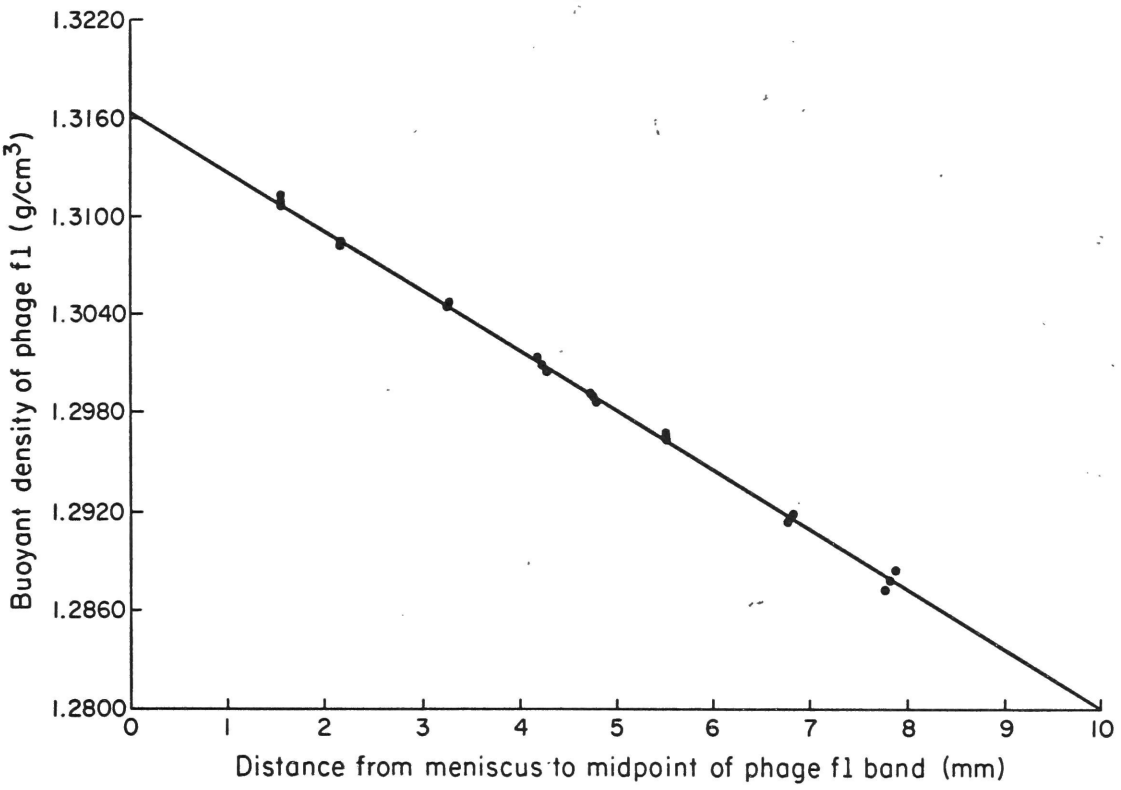

Figure A1: Buoyant density of bacteriophage f1. The buoyant density of phage f1 was determined in CsCl solutions at various mean densities. The vuoyant density is plotted as a function of the distance from the meniscus.

For each set of data, two constant cards must be supplied, the first card must contain the coefficients of beta (β_0, β_1, β_2, β_3) determined by Ifft et al. (1961). The beta coefficients are used in an equation which corrects the density for the activity of the CsCl (Format 41). The second constant card (Format 4) contains the rotor speed in revolutions per minute, the buoyant density of the reference, the optical magnification factor and a control term (GCC). If GCC is greater than 1.00, the computer is directed to calculate the guanine plus cytosine content of the DNA from the equation determined by Schildkraut et al. (1962).

$$\rho_{25} = 0.098 \cdot \text{mole fraction guanine plus cytosine} + 1.660 \text{ g/cm}^3$$

The data is preceded by two control cards. The first card (Format 104) identifies the location where the data is stored on magnetic tape for further calculations, and the second card in alphanumeric format (Format 1040) which identifies the unit of data.

The data from each centrifuge cell is punched onto a single card (Format 2). The sample number and the density or the refractive index of the solution are listed on the card. Then the distance in centimeters between the reference band, the meniscus, and up to four experimental bands and the rotor reference mark, all determined from the tracings are added to the card.

The buoyant density is then calculated according to the option selected. The data on each card is processed individually and the sample number and density of each experimental band is printed. Then the next card is read and processed. If the buoyant density of an experimental band is greater than 0.000, the sample number, its location and density are accumulated into arrays. A blank card at the end of a set of data diverts the program and these arrays are written on magnetic tape and the program is reinitiated for further sets of data. Each data card requires about 5 sec for calculation of the buoyant densities of the experimental bands.

5. Frequency distribution program. The data scored on magnetic tape from the Density Equilibrium Sedimentation Program was used to construct a frequency distribution. This program is listed as Program 3. One constant and control card which contain the file number, tape number, the predicted value for the mode and the width of the class interval (Format 101) is required. The data is then read from magnetic tape and placed into 50 class intervals centered about the expected mode. The number of items in a class interval cannot exceed 30 without overflow. The maximum is located and the standard deviation of the maximum is calculated. Then, the frequency distribution is reconstructed using the maximum for the center and the standard deviation of the maximum for the class interval.

The data were plotted as a frequency diagram. The range of values which bound the class intervals, and the members of the class interval are identified and printed. Then each mode is located and the mean and standard deviation of each mode is determined and printed. Each member of the mode is identified and the distance from the mode in standard deviations was printed. A frequency distribution for 400 items requires about 10 min.

6. Chromatography data program. The Chromatography Data Program was originally written to calculate results from chromatography experiments when more than one isotopic label was used. However, the program was generalized and can process any three continuously distributed variables as a function of fraction number and one linearly distributed variable. Thus this has been very useful to calculate and plot data from equilibrium sedimentation experiments with labeled material. This program is listed as Program 4.

The data are limited to 200 values for each continuously distributed variable and ten values for the linear variable. The scale factors for plotting the continuously distributed variables are determined automatically from the maximum values. The linear variable as written in the program is for CsCl density from the refractive index, but a change in parameter values will allow other linear variables to be used.

There are three options in the program. If sense switch one is on, the two variables in channel one and two are processed; if sense switch one is off, then only one channel selected on the control card is processed. If sense switch two is on, all three channels are processed. If sense switch three is on, the linear variable is processed. One control card is required which contains the location of the data on magnetic tape, and the background count for the data if only one variable is processed (Format 101). One or two constant cards are required which contain the parameters and background for the discriminator ratio method of calculating multiple isotopes (Formats 111 and 1223).

The data for the continuously distributed variables first must be processed by Error Check and Error Correct and stored on magnetic tape. The data for linear variable are punched onto cards and read when required (Format 403). The data are read from magnetic tape and processed according to the options selected. The maximum value of each variable is determined and the scale factors are calculated. The graphs are 8 inches high for the variables and 15 inches long for the fraction number. The scale for each variable is calculated and plotted independently. After each variable is plotted, the program pauses to permit a change of pen color. After finishing the plot, the title is added and the total number of counts for each variable is listed on the graph. The program is reinitiated until all data in a magnetic tape file is completed. A complete plot of three variables, each with two hundred samples, plus the density gradient requires about 7 min. An example of a graph made by the Chromatography Data Program is illustrated in Figure A2.

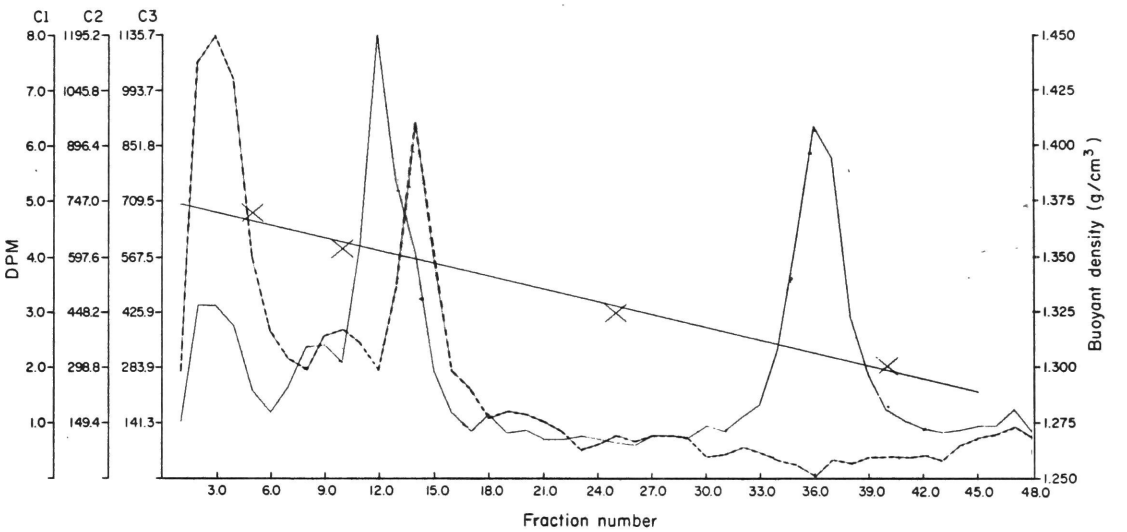

Figure A2: Graph made by Chromatography Data Program. Three isotopic labels from an equilibrium sedimentation gradient are plotted and the density of the gradient derived and plotted from the refractive index of four samples.

COMPUTER PROGRAM 2

```
*     DENSITY EQUILIBRIUM SEDIMENTATION- B. BURLINGHAM-  502 FOUNDERS
      DIMENSION EXPT(5), REXPT(5), DDEN(5), DEXPT(5), GC(5), MAPP(5),
     1ADRESS(500), X(500), LOCATE(500), Z(10),  DOWN(5)
C     MONOCHROMETER-  OMF = 0.1087  UV-  OMF = 0.1153
C     IF GCC POSITIVE--  GC RATIO INCLUDED IN OUTPUT
C     SENSE SWITCH 1-  ON- DENREF= F1, ABSLTE RHO,  OFF- DENREF ON CARD
C     SENSE SWITCH 2, ON= REFRACTIVE INDEX GIVEN
C     SENSE SWITCH 4,  ON= DENREF = MEAN DENSITY
 1123 PAUSE 6
      REWIND 7
      READ 41, BETA0, BETA1, BETA2, BETA3
   41 FORMAT (4F10.5)
      READ 4, OMEGA, DENREF, GCC, OMF
    4 FORMAT (F10.2, 3F10.6)
      OMEGA= OMEGA*6.28318 /60.0000
      READ 104, NFILE, NTAPE
  104 FORMAT (I5, I2)
      READ 1040, (Z(I), I= 1, 10)
 1040 FORMAT (10A6)
      NUMBER = 0
      PRINT 1041, (Z(I), I= 1, 10)
 1041 FORMAT (1H1, 10X, 10A6)
      BOTTOM =  0.8500
   10 READ 2, SAMPLE, AVEDEN, REF, TOP, (EXPT(I), I= 1, 4)
    2 FORMAT (4X, 1A6, 7(F10.4))
      IF (AVEDEN)123,123, 24
   24 IF (SENSE SWITCH 2) 20, 21
   20 AVEDEN= 10.8601*AVEDEN - 13.4974
   21 RREF= 7.300 - REF*OMF
      IF (SENSE SWITCH 1) 22, 23
   22 DENREF=  1.3163 - 0.00364*((TOP-REF)*OMF)
   23 IF (SENSE SWITCH 4) 30, 31
   30 DENREF= AVEDEN
      RREF= 7.300 - (TOP - BOTTOM) * OMF
   31 DO 3, I= 1,5
      IF (EXPT(I)) 6, 6, 5
    6 DEXPT(I)= 00.0000
      GO TO 3
    5 REXPT(I)= 7.3000 - EXPT(I)*OMF
      KK= 1
   54 IF (KK) 3, 51, 52
C     COMPOSITION DENSITY GRADIENT
   52 BETA= (BETA0 +(BETA1*AVEDEN) + (BETA2*AVEDEN**2) + (BETA3*AVEDEN**
     13))*1000000000.0
      GO TO 53
   51 BETA= (BETA0 + (BETA1*DEXPT(I))+(BETA2*DEXPT(I)**2) + (BETA3*DEXPT
     1(I)**3))*1000000000.0
   53 DDEN(I)= OMEGA**2.00/(2.000*BETA)* (REXPT(I)**2.00 - RREF**2.00)
      DEXPT(I)= DENREF + DDEN(I)
      KK= KK - 1
      GO TO 54
    3 CONTINUE
      PRINT 7, SAMPLE,(DEXPT(I), I= 1, 4)
    7  FORMAT ( 1H0, 10X, 1A6, 10X, 4(F10.5, 5X))
      DO 72, I= 1, 5
   72 DOWN(I) = 0.0
      DO 71, I= 1, 5
   71 DOWN(I)= (TOP - EXPT(I)) * OMF
      PRINT 73, (DOWN(I), I= 1, 4)
   73 FORMAT (29X, 4(F10.4, 5X))
      DO 107, I= 1, 5
      IF( DEXPT(I)) 107, 107, 108
```

```
  108 NUMBER = NUMBER + 1
      ADRESS(NUMBER) = SAMPLE
      LOCATE(NUMBER)= I
      X(NUMBER) = DEXPT(I)
  107 CONTINUE
      IF (GCC) 10, 10, 8
    8 DO 80, I= 1, 5
      GC(I)= (DEXPT(I) - 1.6600)/ 0.0980
      IF (GC(I)) 82, 80, 80
   82 GC(I)= 0.0
   80 CONTINUE
      PRINT 81, (GC(I), I= 1, 5)
   81 FORMAT (26X, 5(F10.5, 5X))
    9 GO TO 10
  123 IF (NFILE) 235, 235, 236
  236 I= XFILEF (7, NFILE)
  235 WRITE OUTPUT TAPE 7, 231, (Z(I), I= 1, 10), NUMBER
  231 FORMAT ( 10A6, I3)
      IF (NUMBER - 500) 2310, 2310, 123
 2310 WRITE OUTPUT TAPE 7, 232, (ADRESS(I), X(I), LOCATE(I), I=1,NUMBER)
  232 FORMAT (A6, F12.7, I2)
      ENDFILE 7
      PRINT 233, (Z(I), I= 1, 10), NUMBER, NFILE, NTAPE
  233 FORMAT (1H1, 10X, 10A6, 10X, I3, 20X, 4HFILE, I3, 2X, 4HTAPE, I2)
      PRINT 234, (ADRESS(I), LOCATE(I), X(I), I= 1, NUMBER)
  234 FORMAT (10X, A6, 2X, I2, 5X, F12.7)
      REWIND 7
      GO TO 1123
      END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
KK	1	2 7533	NOT USED
NUMBER	1	2 7632	1 3220
I	1	2 7637	1 3230
NTAPE	1	2 7645	NOT USED
NFILE	1	2 7647	NOT USED
IO	0	3 7775	1 3240

FLOATING POINT VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION
BETA	1	2 7530
RREF	1	2 7551
TOP	1	2 7576
REF	1	2 7601
AVEDEN	1	2 7604
SAMPLE	1	2 7607
BOTTOM	1	2 7615
OMF	1	2 7667
GCC	1	2 7672
DENREF	1	2 7675
OMEGA	1	2 7700
BETA3	1	2 7707
BETA2	1	2 7712
BETA1	1	2 7715
BETA0	1	2 7720

INTEGER ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
LOCATE	1	3 0000	1		
MAPP	1	3 7640	1		

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
DOWN	1	2 7723	1		
Z	1	2 7742	1		
X	1	3 1750	1		
ADRESS	1	3 4704	1		
GC	1	3 7652	1		
DEXPT	1	3 7671	1		
DDEN	1	3 7710	1		
REXPT	1	3 7727	1		
EXPT	1	3 7746	1		

CONSTANTS

VALUE	OBJECT CODE LOCATION
7	2 7440
0.98000000 E-01	2 7454
0.16600000 E 01	2 7457
0.20000000 E 01	2 7516
0.10000000 E 10	2 7521

3	2 7524
2	2 7526
0.0	2 7535
0.36400000 E-02	2 7540
0.13163000 E 01	2 7543
0.73000000 E 01	2 7546
0.13497400 E 02	2 7554
0.10860100 E 02	2 7557
4	2 7574
0.85000000 E 00	2 7612
0	2 7630
0.60000000 E 02	2 7651
0.62831800 E 01	2 7654
10	3 7765
500	3 7767
5	3 7771
1	3 7773

STATEMENT NUMBERS

IDENT	LEVEL	OBJECT CODE LOCATION
2310	1	1 2774
236	1	1 2710
235	1	1 2723
9	1	1 2677
82	1	1 2623
80	1	1 2631
8	1	1 2562
108	1	1 2507
107	1	1 2543
71	1	1 2400
72	1	1 2344
53	1	1 2177
52	1	1 2103
51	1	1 2141
54	1	1 2074
5	1	1 2054
6	1	1 2044
3	1	1 2256
31	1	1 2015
30	1	1 1776
23	1	1 1772
22	1	1 1755
21	1	1 1741
20	1	1 1731
24	1	1 1725
123	1	1 2701
10	1	1 1650
1123	1	1 1461
0	1	1 1461

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION
234	1	2 7347
233	1	2 7365
232	1	2 7422
231	1	2 7432
81	1	2 7442
73	1	2 7462

```
7               1               2 7474
2               1               2 7562
1041            1               2 7620
1040            1               2 7634
104             1               2 7641
4               1               2 7657
41              1               2 7703

LIBRARY FUNCTIONS
  IDENT    LEVEL    OBJECT CODE LOCATION

  (16120                        0 7264
  (R405I                        0 7340
  (R162I                        0 7424
  (W1620                        1 0001
  INPUT                         1 0163
  A**8                          1 0647
  A**I                          1 0713
  EXPF                          1 1003
  LOGF                          1 1223
  XFILEF                        1 1401

ERASABLE STORAGE      1 3250 TO 2 7347
```

COMPUTER PROGRAM 3

```
*     FREQUENCY DISTRIBUTION- 8. BURLINGHAM-  502 FOUNDERS
      DIMENSION ADRESS(400), X(400), Z(10), LOCATE(400), MATRIX( 50,30),
     1MM (50), MODE(20)
  110 PAUSE 1
      REWIND 7
      KOUNT = 0
      READ 101,  NFILE, NTAPE, TXBAR, TCLASS
  101 FORMAT (I5, I2, 2F10.5)
      IF (NFILE) 500 , 500, 501
  501 I= XFILEF (7, NFILE)
  500 READ INPUT TAPE 7, 1, ( Z(I), I= 1, 10), NUMBER
    1 FORMAT (10A6, I3)
      READ INPUT TAPE 7, 2, (ADRESS(I), X(I), LOCATE(I), I= 1, NUMBER)
    2 FORMAT (A6, F12.7, I2)
   11 DO 3, J= 1, 30
      DO 3, I= 1, 50
    3 MATRIX(I, J) = 0
      BOTTOM = 0.0
      TOP = TXBAR - TCLASS*25.0
      DO 31, I= 1, 50
       M= 1
      DO 34, J= 1, NUMBER
      IF (X(J) - BOTTOM)  34, 34, 33
   33 IF (X(J) - TOP) 35, 35, 34
   35 MATRIX (I, M) = J
      M= M + 1
   34 CONTINUE
      BOTTOM = TOP
      TOP = TOP + TCLASS
   31 CONTINUE
C     ALL VALUES ASSIGNED TO TEMPORARY CLASS INTERVALS
C     FIND MODE
      DO 5, I= 1, 50
      M= 0
      DO 51, J= 1, 30
      IF (MATRIX( I, J)) 51, 51, 52
   52  M= M + 1
   51 CONTINUE
      MM(I)= M
    5 CONTINUE
      M= 0
      DO 61, I = 5, 45
      IF (MM(I) - M) 61, 62, 62
   62 M= MM(I)
      K= I
   61 CONTINUE
      IF (KOUNT) 110, 111, 112
C     K= MODE, FIND SIGMA OF MODE-
  111 JJ= K
      DO 7, I= 1, 10
       KM =  K + I
      IF (MM(KM)  - MM(JJ)) 71, 71, 72
   71  JJ= KM
    7 CONTINUE
   72 GO TO 8
    8 J= K
      DO 81, I= 1, 10
       KM= K - I
      IF (MM(KM)     - MM(J)) 82, 82, 83
   82 J = KM
   81 CONTINUE
   83  GO TO 90
```

```
C       WIDTH OF TEMPORARY MOD = J TO JJ
   90 SUMX = 0.0
      N= 0
      DO 9, I= J, JJ
      DO 9,  M= 1, 30
      K= MATRIX( I, M)
      IF (K) 9, 9, 91
   91 SUMX= SUMX + X(K)
      N= N + 1
    9 CONTINUE
      AVE= SUMX/N*1.00
      VARY = 0.0
      N= 0
      DO 10, I= J, JJ
      DO 10, M= 1, 30
      K= MATRIX(I, M)
      IF (K) 10, 10, 1010
 1010 VARY= VARY + (X(K) - AVE) **2.0
      N= N + 1
   10 CONTINUE
      SIGMA = SQRTF (VARY/(N * 1.00))
C       REARRANGE FREQUENCY DISTRIBUTION WITH SIGMA OF MODE
      TXBAR= AVE
      TCLASS = SIGMA
      KOUNT = 1
C       FIND NEW FREQUENCY DISTRIBUTION
      GO TO 11
C       NEW FREQUENCY DISTRIBUTION GENERATED
C       OUTPUT OF TABULAR DATA
  112 PRINT 1120, ( Z(I), I= 1, 10), NUMBER, NFILE, NTAPE
 1120 FORMAT ( 1H1, 10X, 10A6, 10X, I4, 10X, 2I5)
      PRINT 1121
 1121 FORMAT ( 1H0,  5X, 14HCLASS INTERVAL, 10X, 17HINTERVAL MIDPOINT,
     110X, 18HINTERVAL FREQUENCY, 10X, 17HPERCENT FREQUENCY, 5X, 8HINTER
     2VAL)
      BOTTOM= 0
      TOP= TXBAR - 25.0*TCLASS
      DO 13, I= 1, 50
      HALF = (BOTTOM + TOP) / 2.00
      PERCE T= MM(I)/NUMBER*1.000
      PRINT 131,  BOTTOM, TOP, HALF, MM(I), PERCET,  I
  131 FORMAT(1H0,  5X, F10.5, 1X, 1H-, F10.5,15X,F10.5,15X,I4,20X, F8.3,
     110  X, I4)
      PRINT 1310
 1310 FORMAT (50X, 20HMEMBERS OF THE CLASS)
      PRINT 1311
 1311 FORMAT (50X, 4HCELL, 5X, 8HLOCATION, 10X, 7HDENSITY)
      DO 1314, M= 1,30
      IF (MATRIX (I, M))1314, 1314, 1315
 1315 J= MATRIX(I,M)
      PRINT 1312, ADRESS(J), LOCATE(J), X(J)
 1312 FORMAT (50X, A6, 5X, I3, 10X, F12.5)
 1314 CONTINUE
      BOTTOM = TOP
   13 TOP = TOP + TCLASS
C       OUTPUT TO PLOTTER
      TOP= TXBAR - 26.0*TCLASS
      Q= PLOTF ( 5.0, (SIGMA*5.0), 1)
      Q = PLOTF (0.0, -TOP, 2)
      START = SIGMA *100.0
      Q= PLOTF (0.0, START, 3)
      Q= PLOTF (0.0, - TOP, 2)
```

```
      Q= PLOTF (0.0, -TOP, 3)
      Q= PLOTF (0.0, -TOP, 4)
      DO 14, I= 1, 6
      XX= 5.0*I
      Q= PLOTF (XX, -TOP, 4)
      Q= PLOTF (XX, -(TOP - SIGMA), 3)
      Q= PLOTF (XX, -(TOP - SIGMA*3.0), 3)
      PRINT (PLOTTA) 141, XX
  141 FORMAT (3H6 1, F5.1)
      Q= PLOTF(XX, -(TOP-SIGMA), 3)
      Q= PLOTF (XX, -(TOP- SIGMA), 4)
   14 Q= PLOTF (XX, - TOP, 4)
      Q= PLOTF( 0.0, - TOP, 4)
      DO 15, I= 1, 10
      Y= (TOP + 5.00*SIGMA*I) *(-1.000)
      YY= - Y
      Q= PLOTF (0.0, Y, 4)
      Q= PLOTF ( -1.0, Y, 4)
      Q= PLOTF (-1.0, Y, 3)
      Q= PLOTF ( -2.0, (Y + 2.0 * SIGMA), 3)
      PRINT (PLOTTA) 142, YY
  142 FORMAT (3H6 1, F8.5)
      Q= PLOTF (-1.0, Y, 3)
      Q= PLOTF (-1.0, Y, 4)
   15 Q= PLOTF( 0.0, Y, 4)
      Q= PLOTF (0.0, -TOP, 4)
      Q= PLOTF (0.0, -TOP, 3)
      DO 115,  I= 1, 49
      Y= (TOP + SIGMA*I)
      Y= -Y
      Q= PLOTF (0.0, Y, 4)
      Q= PLOTF (-0.5, Y, 4)
  115 Q= PLOTF (0.0, Y, 4)
      Q= PLOTF (0.0, Y, 3)
      Q= PLOTF (-3.0, -TXBAR, 3)
      PRINT ( PLOTTA) 151
  151 FORMAT ( 3H6 1, 14HCLASS INTERVAL)
      Q= PLOTF ( 0.0, - TOP, 3)
      Y= - TOP
      DO 16, I= 1, 50
      XX= MM(I)*1.00
      Q= PLOTF (XX, Y, 4)
      Y= Y- SIGMA
   16 Q= PLOTF (XX, Y, 4)
      Q= PLOTF (XX, Y, 3)
      TOP = TOP - SIGMA
      Q= PLOTF ( 30.0, -TOP, 3)
      PRINT (PLOTTA) 152, (Z(I), I= 1, 8)
  152 FORMAT ( 3H6 1, 8A6)
      Q= PLOTF (28.0, - TOP, 3)
      PRINT (PLOTTA) 153, (Z(I), I= 9,10), NUMBER, NFILE, NTAPE
  153 FORMAT (3H6 1, 2A6, 10X, I4, 2I3)
      Q= PLOTF (28.0, - TOP, 3)
      Q= PLOTF (0.0,-TOP, 3)
C     PLOT OUTPUT COMPLETE
C     FIND MODES, MEANS, STANDARD DEVIATIONS
      K = 0
      DO 161, I= 2, 49
       KM = I - 1
       KMM=  I  + 1
      IF(MM(I) -MM(KM))  161, 161, 162
  162 IF(MM(I) -MM(KMM))  161, 161, 163
```

```
  163 K= K + 1
      MODE(K) = I
  161 CONTINUE
C     MODES FOUND
      PRINT 160
  160 FORMAT ( 1H1, 10X, 7HMODE AT, 10X, 4HMEAN, 10X, 5HSIGMA)
      DO 17, L = 1 , K
      KK = 1
      J = MODE(L)
      DO 171, I = 1, KK
      IF (MM(J) - MM(J - 1)) 171, 172, 172
  172 J= J - 1
      KK= KK + 1
  171 CONTINUE
      JJ = MODE(L)
      KK= 1
      DO 173, I= 1, KK
      IF (MM(JJ) - MM(JJ + 1)) 173, 174, 174
  174 JJ= JJ + 1
      KK= KK + 1
  173  CONTINUE
      SUMX= 0.0
      N= 0
      DO 175, I= J, JJ
      DO 175, K= 1, 30
      M= MATRIX ( I, K)
      IF (M)  175, 175, 176
  176 SUMX= SUMX + X(M)
      N= N + 1
  175 CONTINUE
      XBAR= SUMX/N*1.00
      VARY= 0.0
      N= 0
      DO 177, I= J, JJ
      DO 177, K= 1, 30
      M= MATRIX (I, K)
      IF (M) 177, 177, 178
  178 VARY= VARY + (X(M) - XBAR) ** 2.00
      N= N + 1
  177 CONTINUE
      SIGMA= SQRTF (VARY/N*1.00)
      PRINT 1771, MODE(L), XBAR, SIGMA
 1771 FORMAT ( 1H0, 10X, I3, 10X, F10.6, 10X, F12.8)
      PRINT 1772
 1772 FORMAT (1H0, 40X, 7HADDRESS, 3X, 8HLOCATION, 2X, 7HDENSITY, 3X, 6H
     13SIGMA, 4X, 6H2SIGMA, 4X, 6H1SIGMA)
      TSIG1 = SIGMA + XBAR
      TSIG2 = XBAR + SIGMA* 2.0
      TSIG3= SIGMA*3.0 + XBAR
      BSIG1 = XBAR - SIGMA
      BSIG 2 = XBAR - SIGMA* 2.0
      BSIG3 = XBAR - SIGMA* 3.0
      DO 179, I= J, JJ
      DO 179, K= 1, 30
      M= MATRIX (I, K)
      IF (M) 179, 179, 1791
 1791 IF(TSIG1 - X(M)) 1793, 1794, 1794
 1794 IF(BSIG1- X(M)) 1795, 1795, 1793
 1793 IF ( TSIG2 - X(M)) 1799, 1797, 1797
 1797 IF (BSIG2 - X(M)) 1798, 1798, 1799
 1799 IF(TSIG3- X(M)) 179, 1800, 1800
 1800 IF ( BSIG3 - X(M)) 1801, 1801, 179
```

```
1795 PRINT 1802, ADRESS(M), LOCATE(M), X(M)
1802 FORMAT( 40X, A6, 10X, I2, 10X, F10.6, 10X 1HX)
     GO TO 179
1798  PRINT 1803, ADRESS(M), LOCATE(M), X(M)
1803 FORMAT ( 40X, A6, 10X, I2, 10X, F10.6, 10X, 1HX, 10X, 1HX)
     GO TO 179
1801 PRINT 1804, ADRESS(M), LOCATE(M), X(M)
1804 FORMAT ( 40X, A6, 10X, I2, 10X, F10.6, 10X, 1HX, 10X, 1HX, 10X, 1H
    1X)
 179 CONTINUE
  17  CONTINUE
     REWIND 7
     GO TO 110
     END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
KK	1	2 2606	NOT USED
L	1	2 2610	2 0250
KMM	1	2 2643	2 0256
N	1	2 3307	NOT USED
KM	1	2 3314	2 0264
JJ	1	2 3316	2 0272
K	1	2 3320	2 0300
M	1	2 3326	2 0312
J	1	2 3344	2 0324
NUMBER	1	2 3363	NOT USED
I	1	2 3367	2 0336
NTAPE	1	2 3407	NOT USED
NFILE	1	2 3411	NOT USED
KOUNT	1	2 3415	NOT USED
IO	0	3 7775	2 0354

FLOATING POINT VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION
BSIG3	1	2 2456
BSIG2	1	2 2461
BSIG1	1	2 2464
TSIG3	1	2 2467
TSIG2	1	2 2472
TSIG1	1	2 2475
XBAR	1	2 2603
YY	1	2 2735
Y	1	2 2740
XX	1	2 2755
START	1	2 2771
Q	1	2 3001
PERCET	1	2 3137
HALF	1	2 3142
SIGMA	1	2 3270
VARY	1	2 3276
AVE	1	2 3304
SUMX	1	2 3311
TOP	1	2 3333
BOTTOM	1	2 3341
TCLASS	1	2 3401
TXBAR	1	2 3404

INTEGER ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
MODE	1	2 3417	1		
MM	1	2 3467	1		
MATRIX	1	2 3633	2	50	
LOCATE	1	3 1523	1		

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2

Z	1	3 3163	1
X	1	3 3221	1
ADRESS	1	3 5501	1

CONSTANTS

VALUE	OBJECT CODE LOCATION
9	2 2662
0.28000000 E 02	2 2664
8	2 2676
0.30000000 E 02	2 2700
0.50000000 E 00	2 2721
49	2 2724
0.30000000 E 01	2 2752
6	2 2760
4	2 2762
3	2 2764
0.10000000 E 03	2 2766
2	2 2774
0.50000000 E 01	2 2776
0.26000000 E 02	2 3004
0.20000000 E 01	2 3273
0.10000000 E 01	2 3301
45	2 3322
5	2 3324
0.25000000 E 02	2 3330
0.0	2 3336
7	2 3365
0	2 3413
20	3 7761
30	3 7763
50	3 7765
10	3 7767
400	3 7771
1	3 7773

STATEMENT NUMBERS

IDENT	LEVEL	OBJECT CODE LOCATION
1801	1	2 0061
1800	1	2 0002
1798	1	2 0037
1797	1	1 7753
1799	1	1 7766
1795	1	2 0015
1794	1	1 7725
1793	1	1 7740
1791	1	1 7712
179	1	2 0101
178	1	1 7462
177	1	1 7511
176	1	1 7324
175	1	1 7344
174	1	1 7207
173	1	1 7227
172	1	1 7110
171	1	1 7130
17	1	2 0121
163	1	1 6771
162	1	1 6754

161	1	1 7007
16	1	1 6443
115	1	1 6273
15	1	1 6134
14	1	1 5711
1315	1	1 5332
1314	1	1 5362
13	1	1 5376
1010	1	1 5024
10	1	1 5053
91	1	1 4666
9	1	1 4706
90	1	1 4601
83	1	1 4577
82	1	1 4561
81	1	1 4567
8	1	1 4510
72	1	1 4506
71	1	1 4470
7	1	1 4476
112	1	1 5124
111	1	1 4417
62	1	1 4362
61	1	1 4400
52	1	1 4265
51	1	1 4275
5	1	1 4313
35	1	1 4142
33	1	1 4127
34	1	1 4160
31	1	1 4202
3	1	1 4010
11	1	1 3754
501	1	1 3631
500	1	1 3644
110	1	1 3567
0	1	1 3567

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION
1804	1	2 2341
1803	1	2 2400
1802	1	2 2432
1772	1	2 2500
1771	1	2 2557
160	1	2 2612
153	1	2 2645
152	1	2 2667
151	1	2 2703
142	1	2 2726
141	1	2 2743
1312	1	2 3007
1311	1	2 3026
1310	1	2 3055
131	1	2 3074
1121	1	2 3145
1120	1	2 3246
2	1	2 3346
1	1	2 3356

```
  101          1            2 3371

LIBRARY FUNCTIONS
   IDENT    LEVEL    OBJECT CODE LOCATION

   (16120                   0 7264
   (R405I                   0 7340
   (R162I                   0 7424
   PLOTTA                   1 0001
   INPUT                    1 1725
   A**R                     1 2411
   EXPF                     1 2455
   LOGF                     1 2675
   SQRTF                    1 3053
   PLOTF                    1 3203
   XFILEF                   1 3507


ERASABLE STORAGE      2 0366 TO 2 2341
```

COMPUTER PROGRAM 4

```
*       CHROMATOGRAPHY DATA-  B. BURLINGHAM- 502 FOUNDERS
        DIMENSION Z(10), C1(200), C2(200), C3(200), A(200), RHO(10), ITEM(
       110)
      1 PAUSE 1
        REWIND 7
        READ 101, NUMB, NUMBER, KOLUMN, NSTART, BKG
  101 FORMAT (I3, 2I1, I3, F10.3)
C       ALWAYS INPUT DATA ONTO TAPE IN THREE CHANNELS
C       SELECT 1 ON- DOUBLE LABEL, C1 + C2
C       SELECT 2 ON-  TRIPLE LABEL     1 MUST ALSO BE ON
C       SELECT 4 ON- DENSITY PLOTTED, MUST HAVE THREE CARDS PRESENT
        IF (SENSE SWITCH 1) 112, 113
  112 READ 111, AA, B, HC, CC, BGH, BGC, EH, EC
  111 FORMAT (8F10.5)
        IF (SENSE SWITCH 2) 114, 113
  114 READ 1223, BGX, EX, CX, HX, XCX
 1223 FORMAT (5F10.5)
  113 I= XFILEF (7, NUMB)
C       NUMB= FILE NO, NUMBER= TAPE, KOLUMN= COLUMN FOR INFO, NSTART= BEGI
 1001 READ INPUT TAPE 7, 2, (Z(I), I= 1, 10), N, ISTOP
    2 FORMAT (10A6, I3, 2X, I8)
        XPLOT = 0.0
        READ INPUT TAPE 7, 3, (C1(I), C2(I), C3(I), I= 1, N)
    3 FORMAT (3F12.3)
        IF (SENSE SWITCH 4) 401, 402
  401 READ 403, (ITEM(I), RHO(I), I= 1, 10)
  403 FORMAT (4(I3, 7X, F10.6))
        NRHO = 0
        DO 404,  I= 1, 10
        IF (RHO(I)) 404, 404, 405
  405 RHO(I) = 10.8601*RHO(I) - 13.4974
        NRHO = NRHO + 1
  404 CONTINUE
  402 KOUNT = 1
        IF (SENSE SWITCH 2) 201, 202
  201 DO 203, I= 1, N
        C1(I)= (C1(I) - HX* C3(I))
        C2(I)= (C2(I) - CX*C3(I))
  203 C3(I)=((C3(I) -(XCX*C2(I)))-BGX)/EX
        GO TO 204
  202 IF(SENSE SWITCH  1) 204, 205
  204 DO 206, I= 1, N
        H= C1(I)
        C= C2(I)
        C1(I)=(((B*H-C)/B-AA)/EH)  - BGH
  206 C2(I)=(((B*(C- AA*H))/B-AA)/EC ) - BGC
        IF  (SENSE SWITCH 1)  207, 208
  207 KOLUMN= 2
  208 IF (SENSE SWITCH 2) 209, 205
  209 KOLUMN= 3
  205 PRINT 300, (Z(I), I= 1, 10), N, NUMB, NUMBER
  300 FORMAT ( 1H1, 1X, 10A6, 10X, 3I5)
        PRINT 301
  301 FORMAT (10X, 10HH3-CHANNEL, 10X, 11HC14-CHANNEL, 10X, 11HP32-CHANN
     1EL)
        KSTART = NSTART + 1
        NEND= NSTART + N
        PRINT 302, ( I, C1(I), C2(I), C3(I), I= KSTART, NEND)
  302 FORMAT (3X, I5, 2X, 3(F10.1, 10X))
  215 AMAX= 0.0
        KOUNT = KOUNT + 1
        GO TO (4, 5, 6) KOLUMN
```

```
    4 DO 41, I= 1, N
   41 A(I)= C1(I)
      GO TO 7
    5 DO 51, I= 1, N
   51 A(I)= C2(I)
      GO TO 7
    6 DO 61, I= 1, N
   61 A(I)= C3(I)
    7 SUMA= 0.0
      DO 8, I= 1, N
      A(I)= A(I) - BKG
      IF (A(I)) 71, 72, 72
   71 A(I)= 0.0
   72 SUMA= SUMA + A(I)
      IF (A(I) - AMAX) 8, 8, 9
    9 AMAX= A(I)
    8 CONTINUE
       IF (AMAX) 81, 81, 82
   81 AMAX = 8.0
   82 YSTART= NSTART * 1.00
      X= AMAX/8.0
      NY= N/14
      Y= NY*1.00
      Q= PLOTF (X, Y, 1)
      END = YSTART + 16.00 * Y
      ORIGIN= YSTART + Y*16.0 + XPLOT*Y
      Q= PLOTF(0.0,  YSTART, 2)
      Q= PLOTF (0.0, ORIGIN, 3)
      Q= PLOTF (0.0,-YSTART, 2)
      Q= PLOTF (0.0, -YSTART, 4)
      DO 10, I= 1, 8
      XAX= X*I
      Q= PLOTF (XAX, -YSTART, 4)
      Q= PLOTF (XAX, -(YSTART - 0.25*Y), 4)
      Q= PLOTF ( XAX, -(YSTART - 0.25*Y), 3)
      Q= PLOTF (XAX, -(YSTART - 1.00*Y), 3)
      PRINT (PLOTTA) 11, XAX
   11 FORMAT ( 3H6 1,F10.1)
      Q=PLOTF (XAX, -(YSTART - 0.25*Y), 3)
      Q=PLOTF (XAX, -(YSTART - 0.25*Y), 4)
   10 Q= PLOTF (XAX, -YSTART, 4)
      Q= PLOTF (XAX, -YSTART, 3)
      XAXX = XAX + X * 0.25
      Q= PLOTF (XAXX, - YSTART, 3)
      PRINT(PLOTTA) 122, KOLUMN
  122 FORMAT (3H6 1, 1HC, I1)
      Q= PLOTF (XAX, -YSTART, 3)
      Q= PLOTF (0.0, -YSTART, 4)
      Q= PLOTF( 0.0, -YSTART, 3)
      IF (SENSE SWITCH 1) 1221, 1222
 1222  IF (SENSE SWITCH 2)  1221, 1224
 1224 Q= PLOTF((4.0*X), -(YSTART-1.5*Y), 3)
      PRINT (PLOTTA) 100
  100 FORMAT (3H6 1, 3HCPM)
 1221 Q=PLOTF  (0.0, -YSTART, 3)
      IF (XPLOT -1.0)  122, 121, 121
  122 DO 12, I= 1, 16
      YAX=  YSTART + Y*I
      Q= PLOTF (0.0, -YAX, 4)
      Q= PLOTF (-0.25*X, -YAX, 4)
      Q= PLOTF (-0.25*X, -YAX, 3)
      Q= PLOTF (-0.50*X, -(YAX - Y*0.3), 3)
```

```
      PRINT (PLOTTA) 13, YAX
   13 FORMAT (3H6 1, F6.1)
      Q= PLOTF (-0.25*X, -YAX, 3)
      Q= PLOTF (-0.25*X, -YAX, 4)
   12 Q= PLOTF (0.0,     -YAX, 4)
      Q= PLOTF (0.0,-YSTART , 4)
      Q= PLOTF (0.0,-YSTART , 3)
      Q= PLOTF ( -X, -(YSTART + 6.0*Y), 3)
      PRINT (PLOTTA) 120
  120 FORMAT ( 3H6 1, 15HFRACTION NUMBER)
  121 YAX = YSTART + XPLOT* Y
      Q= PLOTF (0.0, -YAX, 3)
      Q= PLOTF (0.0, - YSTART, 2)
      Q= PLOTF (0.0, -YSTART, 3)
      YVALUE= NSTART * 1.0 + 1.0
      Q= PLOTF (A(1),-YVALUE, 3)
      DO 14, I= 1, N
      YVALUE =  NSTART  + I
   14 Q= PLOTF ( A(I), -YVALUE, 4)
      Q= PLOTF ( A(I), -YVALUE, 3)
      XPLOT = XPLOT + 1.0
      IF (SENSE SWITCH 1) 220, 211
  211 IF (SENSE SWITCH 2) 220, 212
  220 KOLUMN = KOLUMN - 1
      Q= PLOTF (0.0, - END, 3)
      PAUSE 10
      IF (KOLUMN) 212, 212, 213
  213 SUMC= SUMA
      IF (KOLUMN - 1) 212, 215, 214
  214 SUMX= SUMA
      GO TO 215
  212 Q= PLOTF (X*8.5, -(YSTART + Y), 3)
      PRINT (PLOTTA) 140, (Z(I), I= 1, 8)
  140 FORMAT (3H6 1, 8A6)
      Q= PLOTF (X*8.25,  -(YSTART+ Y) , 3)
      IF(SENSE SWITCH 2) 240, 2414
  240 PRINT (PLOTTA) 143, (Z(I), I= 9, 10), NUMB, NUMBER, SUMA, SUMC, SU
     1MX
  143 FORMAT (3H6 1, 2A6, 2(I4, 1H,), 3F8.0)
      GO TO 241
 2414 IF (SENSE SWITCH 1) 230, 231
  230 PRINT (PLOTTA) 142, (Z(I), I= 9, 10), NUMB, NUMBER, SUMA, SUMC
  142 FORMAT (3H6 1, 2A6, 2(I4, 1H,), 2F10.1)
      GO TO 241
  231 PRINT (PLOTTA) 141, (Z(I), I= 9, 10), NUMB, NUMBER, KOLUMN, SUMA
  141 FORMAT (3H6 1, 2A6, 3(I4,  1H,), F10.1)
  241 IF (SENSE SWITCH 4)  406, 242
  242 YVALUE= YSTART + Y*4.0
      Q= PLOTF (0.0,  YVALUE, 3)
      GO TO 412
  406 Q= PLOTF (0.0, -YSTART, 3)
      ORIGIN=  YSTART + 16.0*Y
      Q= PLOTF (0.0250, Y, 1)
C     Q= PLOTF (0.0500, Y, 1)
C     Q= PLOTF (0.1000, Y, 1)
      Q= PLOTF (1.2500, - YSTART, 2)
      Q= PLOTF (1.2500, - YSTART, 3)
      DO 408, I= 1, NRHO
      YITEM1 = ITEM(I)*1.00
      YITEM2 = YITEM1 + Y/ 5.0
      YITEM1 = YITEM1 - Y/5.0
      RHO1 = RHO(I) - 0.004
```

```
      RHO2 = RHO(I) + 0.004
      Q= PLOTF ( RHO1,-YITEM1, 3)
      Q= PLOTF ( RHO 1,-YITEM 1, 4)
      Q= PLOTF (RHO2,-YITEM2, 4)
      Q= PLOTF (RHO2,-YITEM2, 3)
      Q= PLOTF (RHO2,-YITEM1, 3)
      Q= PLOTF (RHO2,-YITEM1, 4)
      Q= PLOTF (RHO1,-YITEM2, 4)
  408 Q= PLOTF (RHO1,-YITEM2, 3)
      END = - ORIGIN
      Q= PLOTF ( 1.2500, END, 3)
      DO 409, I= 1,9
      X= 1.2250 + 0.0250*I
C     X= 1.2000 + 0.05000 * I
C     X= 1.10000 + 0.10000 * I
      Q= PLOTF ( X, END, 4)
      Q= PLOTF ( X, (END - Y/5.0), 4)
      Q= PLOTF ( X, (END - Y/5.0), 3)
      Q=PLOTF (X, (END - Y/2.0), 3)
      PRINT(PLOTTA)410     , X
  410 FORMAT ( 3H6 1, F9.5)
      Q= PLOTF ( X, (END - Y/5.0), 4)
  409 Q= PLOTF ( X, END, 4)
      Q= PLOTF ( 1.2500, END, 4)
      Q= PLOTF (1.2500, END, 3 )
      SUMX = 0.0
      SUMY= 0.0
      SUMXY= 0.0
      SUMX2 = 0.0
      DO 411, I= 1, NRHO
      YITEM= ITEM(I) * 1.000
      SUMX= SUMX + YITEM
      SUMY = SUMY + RHO(I)
      SUMXY = SUMXY + YITEM*RHO(I)
  411 SUMX2 = SUMX2 + YITEM**2.00
      RRHO = NRHO * 1.00
      SLOPE  = (RRHO*SUMXY - SUMX*SUMY)/(RRHO*SUMX2- SUMX**2)
      REMAIN= (SUMY*SUMX2 - SUMXY*SUMX)/(RRHO*SUMX2 - SUMX**2)
      X1 = YSTART  + 1.0
      X2 = YSTART + 15.0*Y
      Y1 = SLOPE * X1 + REMAIN
      Y2 = SLOPE * X2 + REMAIN
      Q= PLOTF ( Y1,-X1, 3)
      Q= PLOTF ( Y1,-X1, 4)
      Q= PLOTF (Y2,-X2,4)
      Q= PLOTF ( Y2,-X2, 3)
      PAUSE 11
      Q= PLOTF (1.2500,  -YSTART, 3)
      Q= PLOTF  (1.2500, (5.0*Y), 3)
  412 IF (ISTOP) 1, 1001, 1011
 1011 REWIND 7
      GO TO 1
      END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
NY	1	3 2567	NOT USED
NEND	1	3 2631	NOT USED
KSTART	1	3 2633	NOT USED

KOUNT	1	3 2723	NOT USED
NRHO	1	3 2735	NOT USED
ISTOP	1	3 2773	NOT USED
N	1	3 2775	NOT USED
I	1	3 3001	2 0141
NSTART	1	3 3077	NOT USED
KOLUMN	1	3 3101	NOT USED
NUMBER	1	3 3103	NOT USED
NUMB	1	3 3105	NOT USED
IO	0	3 7775	2 0151

FLOATING POINT VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION
Y2	1	3 2212
Y1	1	3 2215
X2	1	3 2223
X1	1	3 2226
REMAIN	1	3 2231
SLOPE	1	3 2234
RRHO	1	3 2237
YITEM	1	3 2242
SUMX2	1	3 2245
SUMXY	1	3 2250
SUMY	1	3 2253
RHO2	1	3 2273
RHO1	1	3 2301
YITEM2	1	3 2307
YITEM1	1	3 2312
SUMX	1	3 2421
SUMC	1	3 2424
YVALUE	1	3 2427
YAX	1	3 2470
XAXX	1	3 2522
XAX	1	3 2537
ORIGIN	1	3 2546
END	1	3 2554
Q	1	3 2557
Y	1	3 2562
X	1	3 2571
YSTART	1	3 2577
SUMA	1	3 2605
AMAX	1	3 2610
C	1	3 2715
H	1	3 2720
XPLOT	1	3 2757
XCX	1	3 3007
HX	1	3 3012
CX	1	3 3015
EX	1	3 3020

BGX	1	3 3023			
EC	1	3 3032			
EH	1	3 3035			
BGC	1	3 3040			
BGH	1	3 3043			
CC	1	3 3046			
HC	1	3 3051			
B	1	3 3054			
AA	1	3 3057			
BKG	1	3 3074			

INTEGER ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
ITEM	1	3 3107	1		

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
RHO	1	3 3133	1		
A	1	3 3171	1		
C3	1	3 4321	1		
C2	1	3 5451	1		
C1	1	3 6601	1		
Z	1	3 7731	1		

CONSTANTS

VALUE	OBJECT CODE LOCATION
0.15000000 E 02	3 2220
0.20000000 E 01	3 2265
0.12250000 E 01	3 2270
0.40000000 E-02	3 2276
0.50000000 E 01	3 2304
0.12500000 E 01	3 2315
0.25000000 E-01	3 2320
9	3 2403
0.82500000 E 01	3 2405
0.85000000 E 01	3 2416
0.60000000 E 01	3 2450
0.30000000 E 00	3 2462
0.50000000 E 00	3 2465
16	3 2473
0.15000000 E 01	3 2504
0.40000000 E 01	3 2507
0.25000000 E 00	3 2534
8	3 2542
4	3 2544
0.16000000 E 02	3 2551
14	3 2565
0.10000000 E 01	3 2574
0.80000000 E 01	3 2602
3	3 2711
2	3 2713
0.13497400 E 02	3 2725
0.10860100 E 02	3 2730
0	3 2733
0.0	3 2754
7	3 2777
200	3 7767

	10	3 7771
	1	3 7773

STATEMENT NUMBERS IDENT	LEVEL	OBJECT CODE LOCATION
1011	1	2 0111
411	1	1 7621
409	1	1 7447
408	1	1 7232
412	1	2 0102
242	1	1 6702
406	1	1 6730
231	1	1 6630
230	1	1 6560
241	1	1 6676
2414	1	1 6554
240	1	1 6502
214	1	1 6370
213	1	1 6353
212	1	1 6376
211	1	1 6313
220	1	1 6317
14	1	1 6235
12	1	1 6010
121	1	1 6111
122	1	1 5624
1224	1	1 5550
1222	1	1 5544
1221	1	1 5577
10	1	1 5402
82	1	1 5033
81	1	1 5027
9	1	1 5002
72	1	1 4757
71	1	1 4751
8	1	1 5010
61	1	1 4670
51	1	1 4632
7	1	1 4706
41	1	1 4574
6	1	1 4652
5	1	1 4614
4	1	1 4556
215	1	1 4531
209	1	1 4363
208	1	1 4357
207	1	1 4351
206	1	1 4311
205	1	1 4371
204	1	1 4235
203	1	1 4200
202	1	1 4231
201	1	1 4136
405	1	1 4072
404	1	1 4114
402	1	1 4124
401	1	1 3773
1001	1	1 3647
114	1	1 3616

113	1	1 3634
112	1	1 3566
1	1	1 3527
0	1	1 3527

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION
410	1	3 2256
141	1	3 2323
142	1	3 2343
143	1	3 2363
140	1	3 2410
120	1	3 2432
13	1	3 2453
100	1	3 2475
122	1	3 2512
11	1	3 2525
302	1	3 2613
301	1	3 2635
300	1	3 2674
403	1	3 2737
3	1	3 2750
2	1	3 2762
1223	1	3 3003
111	1	3 3026
101	1	3 3062

LIBRARY FUNCTIONS

IDENT	LEVEL	OBJECT CODE LOCATION
(16120		0 7264
(R405I		0 7340
(R162I		0 7424
PLOTTA		1 0001
INPUT		1 1725
A**B		1 2411
A**I		1 2455
EXPF		1 2545
LOGF		1 2765
PLOTF		1 3143
XFILEF		1 3447

ERASABLE STORAGE 2 0162 TO 3 2212

APPENDIX D: SUPPLEMENTARY INFORMATION FOR ZONAL SEDIMENTATION

1. Computer program for zonal sedimentation data from the analytical ultracentrifuge. The data from band velocity sedimentation determinations were calculated by the Velocity Sedimentation Program listed as Program 5. Sense switch one must be used to select the optical magnification factor for either the monochromater (on) or the direct UV light source (off). Two constant cards are required, one which contains the viscosity, the partial specific volume and density information (Format 41) and one which contains the sample number, the revolutions per minute and the number of determinations to be averaged (Format 5). The experimental data, the time of the photograph and the distance on the tracing from the maximum to the rotor reference mark, was entered on cards (Format 6). The calculations were done according to translations of the equations explained on page 92. The sample number, mean of the determinations for $\underline{S}_{obs}$ and $\underline{S}_{20,w}$ and the standard deviations of these means were calculated and printed. The program was then reinitiated for other sedimentation data using the same solvent. Each sedimentation coefficient derived from 10 measurements was calculated in about 20 sec.

2. Zonal sedimentation in sucrose density gradients

a. Control data for sucrose density gradients. The linearity of the sucrose density gradients was established by adding methyl orange to the 20% sucrose solution. After preparing the 5 to 20% sucrose density gradient, 0.2 ml fractions were collected, and the absorption at 450 nm was determined for each fraction. These data are seen in Figure A3. The sucrose density gradients are stable for at least 48 hr when stored at 4°C.

Since the sucrose gradients were used to assay the size of DNA preparations, the gradient must have no deoxyribonuclease activity. A sample of Ad 2 DNA-^{3}H was incubated with 20% neutral sucrose and the size and distribution pattern of the label was determined. The data from this experiment are seen in Figure A4. Since the size of the DNA did not change

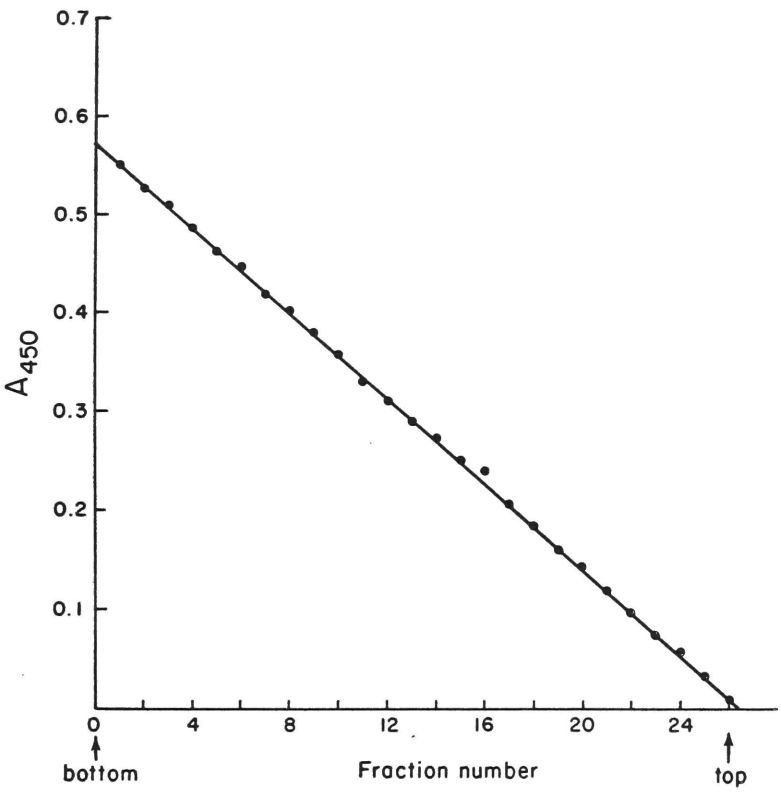

Figure A3: Linearity of neutral sucrose gradients. Methyl orange was added to the 20% neutral sucrose solution. The regular procedure was followed to prepare a 5 to 20% sucrose gradient. After 3 hr of storage, 0.2 ml fractions were collected from the bottom of the tube, 0.8 ml distilled water was added to each fraction and the absorption at 450 nm was determined. A regression line was determined for the data.

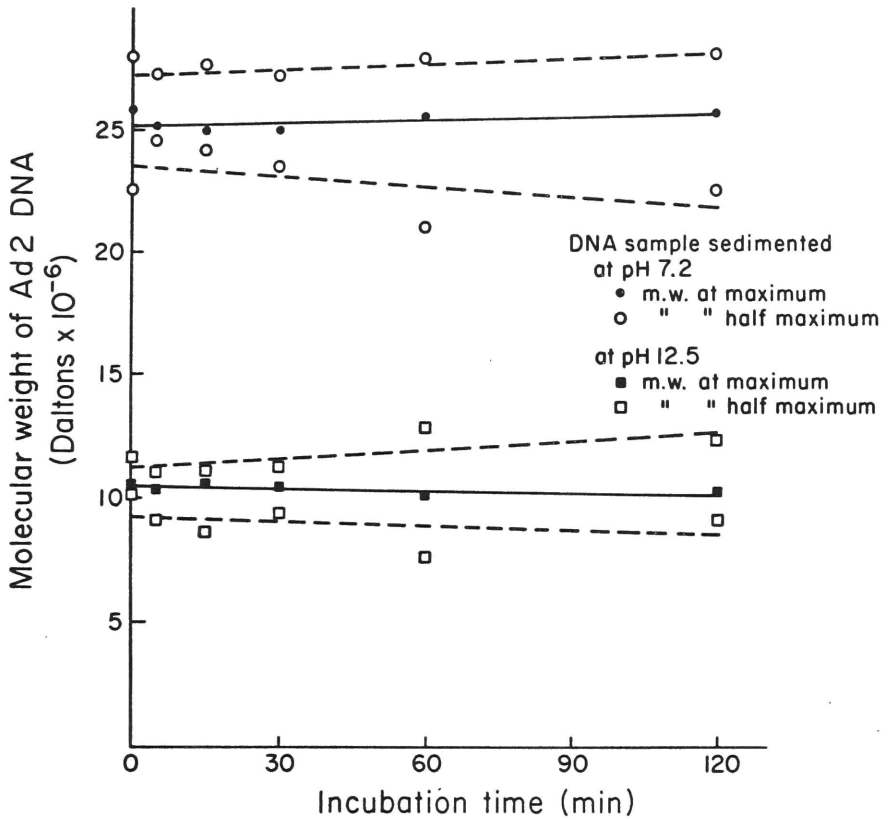

Figure A4: Deoxyribonuclease activity of 20% neutral sucrose solution. A 10 μg sample of Ad 2 DNA-^{3}H was incubated with 20% neutral sucrose. At intervals during the incubation, the size of the DNA was determined in neutral and alkaline sucrose gradients. The data for the molecular weight at the maximum and half maximum are given. Regression lines have been drawn for the data.

during the two hour incubation period, the 20% neutral sucrose solution has no deoxyribonuclease activity.

Since three different rotors were used for sucrose density gradient analysis, the sedimentation in these rotors must be isokinetic if the results are compared and the sedimentation coefficients are to be accurate. Doty et al. (1958) proposed the relationship: $\underline{S} = aM^k$ between the sedimentation coefficients ($\underline{S}$) and the molecular weight (M) of DNA's. deDuve et al. (1959) defined $\underline{S}$ under these conditions as $\underline{S} = \frac{dx/dt}{\omega^2 x}$ where dx/dt is the velocity at which the band of molecules sediments, x is the distance between the band of molecules and the center of rotation, and ω is the angular velocity in radians per second.

Under isokinetic conditions, the velocity (dx/dt) for a single species of molecules is constant and thus independent of x and dependent only on ω. When ω is constant and the conditions are proper for isokinetic sedimentation, the velocity (dx/dt) for a given species of molecules is constant and dependent on the mass and shape of the molecules. Thus the distance (D) a species travels in zone sedimentation is defined by $D = dx/dt \cdot t$, where t is the time of sedimentation at the angular velocity ω.

When two species are compared in the same isokinetic gradient, ω and t, with respect to these two species are constant and the relative distance either species travels is equal to $D = dx/dt \cdot t$ and thus:

$$\underline{S}_1 = \frac{D_1}{\omega^2 t} \quad \text{and} \quad \underline{S}_2 = \frac{D_2}{\omega^2 t} \quad ; \quad \frac{\underline{S}_1}{\underline{S}_2} = \frac{D_1}{D_2}$$

The distance a DNA species sediments in a sucrose density gradient is compared with the distance a marker DNA sediments. Thus the ratio of the experimental species to the marker species becomes:

$$\frac{D_1}{D_2} = \frac{\underline{S}_1}{\underline{S}_2} = \left[\frac{(M_1)}{(M_2)}\right]^K$$

The accuracy of this simple relationship depends on isokinetic conditions, and these conditions must be established for the rotors used. Thus the effect of the gravitational field and the resistance of the gradient must be equal and opposite to give isokinetic sedimentation from the top to the bottom of the tube. The gravitational field ($\underline{g}$) acting on a molecule is defined by $\underline{g} = \omega^2 \cdot x$, and x is constantly increasing during sedimentation. Thus isokinetic conditions can be obtained only if the resistance to sedimentation of the molecule increases at the same rate as the radius of rotation increases.

The viscosity and density, interpolated from tables in deDuve et al. (1959), of 5% and 20% sucrose solution at 4°C are 1.5664 centipoise and 1.0567 g/cm^3 and 3.1236 centipoises and 1.1198 g/cm^3, respectively. The relative resistance to sedimentation, mainly due to frictional resistance and relative decreased mass, from the top to the bottom of the gradient increases by a factor of 2.0. Thus the gravitational field must increase by a factor of 2.0 to maintain isokinetic conditions. Since $\underline{g} = \omega^2 \cdot r$, where r is the radius, and ω of any two radii of a tube is constant, the radius at the bottom must be 2.0 times the radius of the top of the tube to attain isokinetic conditions throughout the gradient.

The data in Table A11 compares the radii of the rotors used. The SW 56 and the SW 39 rotors have the approximate ratio of top to bottom radii to be used with 5 to 20% sucrose gradients. The ratio for the SW 50.1 rotor is too low to be used at isokinetic conditions with 5 to 20% sucrose gradients.

The rate of sedimentation of three different sized DNA samples in these rotors was tested using 5 to 20% sucrose gradients. The rate of sedimentation was determined four times for each DNA sample by varying the time of sedimentation and determining the distance the DNA has sedimented. The time of sedimentation was accurately determined, and acceleration and deacceleration time was measured and one-third of this time was added to the final sedimentation time. Fractions from the gradients were collected and the position of the DNA determined. The point of maximum radioactivity was taken as the center of mass for calculations.

Table A11

Comparison of radii of rotors used[a]

Rotor	Radius of rotation at top (r_t)	Radius of rotation at bottom (r_b)	r_b/r_t
SW 39	4.70	9.80	2.085
SW 50.1	5.97	10.73	1.796
SW 56	5.93	11.64	1.961

[a] Data from Spinco Manual.

The sedimentation data for these three rotors and three DNA samples are shown in Figure A5. The mean and range of the four determinations were plotted. As expected, the sedimentation with 5 to 20% sucrose density gradient in the SW 56 or SW 39 rotor is isokinetic. But sedimentation in the SW 50.1 rotor is not isokinetic and the sedimentation coefficient decreases as the DNA sediments, since the resistance to sedimentation increases faster than the gravitational force. Thus the SW 50.1 was used only for preliminary experiments while the SW 56 or SW 39 rotors were used for accurate determinations. Table A12 compares the distances the three DNA species sedimented with the S values. The SW 39 rotor is the most accurate since the standard deviation is the smallest and the ratios are close to the theoretical. The accuracy of the SW 56 rotor is almost equal to the accuracy of the SW 39 rotor. The SW 56 has the additional advantage that the higher gravitational field separates two DNA species of nearly the same size further in a shorter run and less diffusion results. Both of these produce greater resolution.

Three methods of collecting fractions were evaluated. A 20% sucrose solution which contained DNA-^{3}H was placed in the tube. Fractions were collected from the bottom of the tube by manual or mechanical means or from the top of the tube by pumping a 50% sucrose solution into the bottom of the tube. The radioactivity of each fraction was determined. These data are found in Table A13.

The most accurate method of collecting fractions was by flotation from the top of the tube; however, this method took 20 to 30 min per gradient and thus was not a practical method. The error of collecting fractions manually from the bottom of the gradient was about twice the error when mechanical means were used. Manual collection resulted in a variation which was 3% of the total activity. The manual method was rapid and the error was about equal to the error of calculating the radioactivity of the DNA. Thus fractions from sucrose gradients were routinely collected manually.

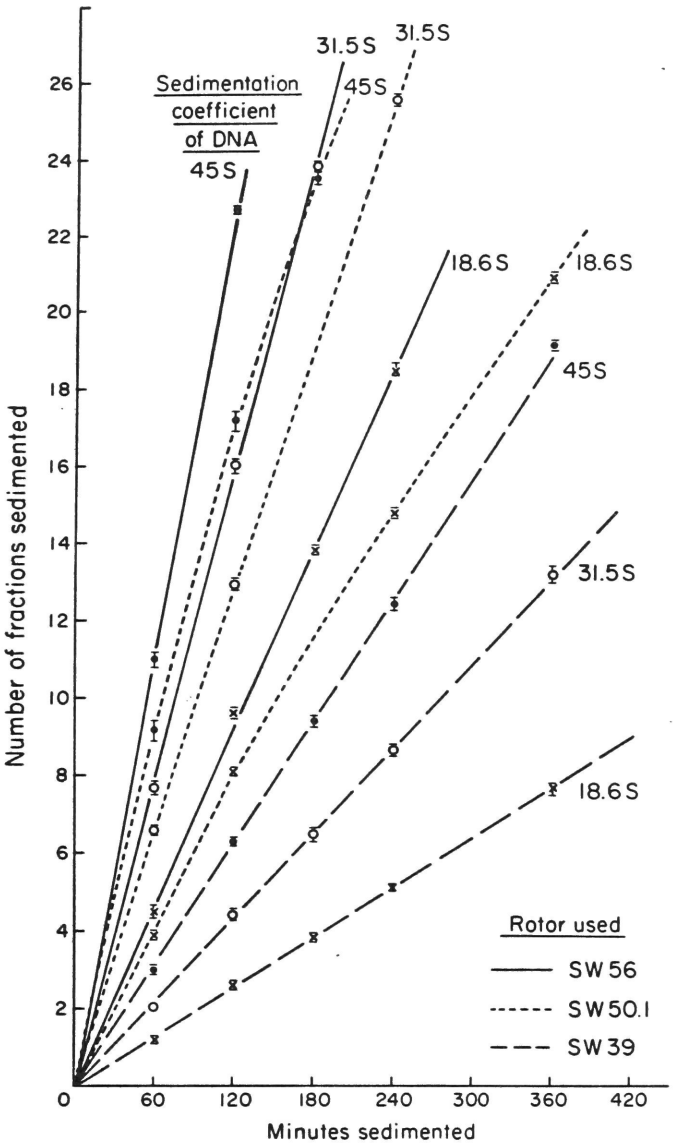

Figure A5: Evaluation of isokinetic sedimentation of DNA in three different rotors. Three different DNA species, 18.6 S, 31.5 S and 45 S were sedimented in neutral sucrose density gradients using three different rotors. The time of sedimentation and the distance sedimented were accurately determined.

Table A12

Comparison of Distances of Three DNA Species Sedimented and the Ratio of Distances for Three Rotors

Rotor	Speed ($\times 10^{-3}$ rpm)	18.6 S DNA dx/dt[a]	$\frac{D^{31.5}}{D^{18.6}}$	31.5 S DNA dx/dt	$\frac{D^{45}}{D^{31.5}}$	45 S DNA dx/dt	$\frac{D^{45}}{D^{18.6}}$
SW 39	33	.257 ± .004	1.716	.441 ± .001	1.433	.632 ± .007	2.459
SW 50.1	50	.767 ± .025	1.916	1.336 ± .032	1.334	1.782 ± .043	2.323
SW 56	56	1.166 ± 0.015	1.709	1.907 ± .0153	1.412	2.693 ± .042	2.413
		$\frac{S^{31.5}}{S^{18.6}} = 1.694$		$\frac{S^{45}}{S^{31.5}} = 1.429$		$\frac{S^{45}}{S^{18.6}} = 2.419$	

[a] Velocity = cm/hr.

[b] Mean and standard deviation.

Table A13

Comparison of Three Methods of Collecting Fractions from Sucrose Gradients

Method of collecting fractions	Mean[a] cpm/fraction	Theoretical[b] standard deviation	Actual[c] standard deviation	% error collection[d]	% error total
Manual collection from bottom	24,179	155.49	1,146.98	4.1	4.7
Automatic collection from bottom	22,072	148.56	897.88	3.3	4.0
Automatic collection from top	23,698	153.94	631.15	2.0	2.6

[a] Mean of three gradients, each gradient had 25 individual fractions.

[b] The theoretical standard deviation is the counting error. This is equal to the square root of the number of disintegrations counted.

[c] The actual standard deviation is composed of the counting error plus the variation in volumes during collection. The standard deviation for the 25 fractions of each gradient was calculated. The value listed is the mean standard deviation of 3 gradients.

[d] Collection error = $\frac{\text{actual standard deviation - theoretical standard deviation}}{\text{mean radioactivity per sample}}$

b. Methods of processing data from sucrose density gradients. All of the programs involved in processing sucrose density gradients were stored in compiled form on magnetic tape and were recalled by the Fortran bootstrap.

1) Error check program. The data from the liquid scintillation counter was automatically punched onto data cards. This process was a rapid and accurate way for transcribing the numerical values; however, two types of errors were introduced. All the data on the card may be entered into wrong columns due to tabulation errors or individual values were entered into improper columns from a failure to output the correct number of preceding zeros. These were the only errors found in periodic spot checks of one hundred or more cards each month.

Two programs were written which detected and corrected these errors before the data were transcribed onto magnetic tape for permanent storage. Error Check, listed as Program 6, was the program which surveyed each card and each number for these errors. The data cards were prepared for each gradient by punching a title card which contained a maximum of 60 characters which identified the data and the number of samples in the gradient (Format 100). The last gradient in a data set had a number greater than 1 in column 71 of the title card as a stop signal.

Error Check is limited to gradients which contain less than 200 samples. The variables are read in alphanumeric format and the presence of tabulation errors are detected and recorded. The presence of illegal blanks are noted for each variable by using Boolean masking statements. The errors were coded by numbers, one number specifying the tabulation errors in the card format and one number for each variable specifying the number of illegal blanks. These numbers specifying the corrections were stored on magnetic tape and the next gradient was read and evaluated for corrections.

2) Error correct program. The errors in the cards were corrected by the Error Correct Program listed as Program 7. It has the same limitations on variables as Error Check. The information which

controls the correction of the data is read from tape, the title card is read and compared with the title card on tape. If the titles are identical, the data are read and corrected by a series of computed go to statements directed by the information from Error Check which was stored on magnetic tape. After the data are corrected, the title card and the corrected card images are stored on magnetic tape. The entire process of correcting the data and storing it on magnetic tape takes about one min per gradient.

3) Scintillation data program. The actual calculations of the sucrose gradient data were done by the Scintillation Data Program listed as Program 8. The number of samples in the gradient is restricted to 40 samples. There are three options available in the program which can be combined to give high versatility. If sense switch one is on, the data are plotted and calculated directly from the corrected data. If sense switch one is off, a third order interpolated calculation and plot are made. If sense switch two is on, no derived data are determined; if sense switch two is off, the sedimentation coefficient, molecular weight and relative area of each maximum is determined. If sense switch four is on, an arithmetic graph of the data is made; if the switch is off, a relative graph of the data is made.

One control card is required for the program (Format 91) which identifies the data processed, the sedimentation coefficient of the marker (determined in the analytical ultracentrifuge) and a control number specifying whether the calculations are for native DNA, denatured DNA or virus particles.

Two constant cards are required. One contains constants for the scale of the arithmetic plot (Format 110) and one contains the constants from the Scintillation Parameter Program specifying the coefficients, corrections, efficiencies and background counts for the calculations (Format 111). Then the title and data are read from magnetic tape. The data are processed by the discriminator ratio method described by Okita et al. (1957). After calculation, the original data and the calculated data for ^{3}H and ^{14}C were printed.

The data are plotted either directly or from a third order interpolation of the data. The interpolation calculates values at one-tenth fraction intervals over the entire gradient. The equation of the curve was derived with the Chebyshev polynomials and expansion using regional data, the last actual data point, the last two interpolated values and the next two actual data points. The matrix generated was solved by simple Gauss elimination with no row interchanges required since there are no possible zero diagonal terms. These points were then plotted and the maxima of the marker and experimental curve were found. The sedimentation coefficient was calculated by the method of Martin and Ames (1961). The molecular weights were calculated by the equations derived by Studier (1965). The area under the curves was determined by integration of each maximum. These values were then printed out and the program reinitiated with the next gradient. An interpolated plot required about 20 min per gradient. The standard error of the mean of replicate determinations was ± 0.12 $\underline{S}$ using interpolated data. An example of the graph of the interpolated relative data is illustrated in Figure A6.

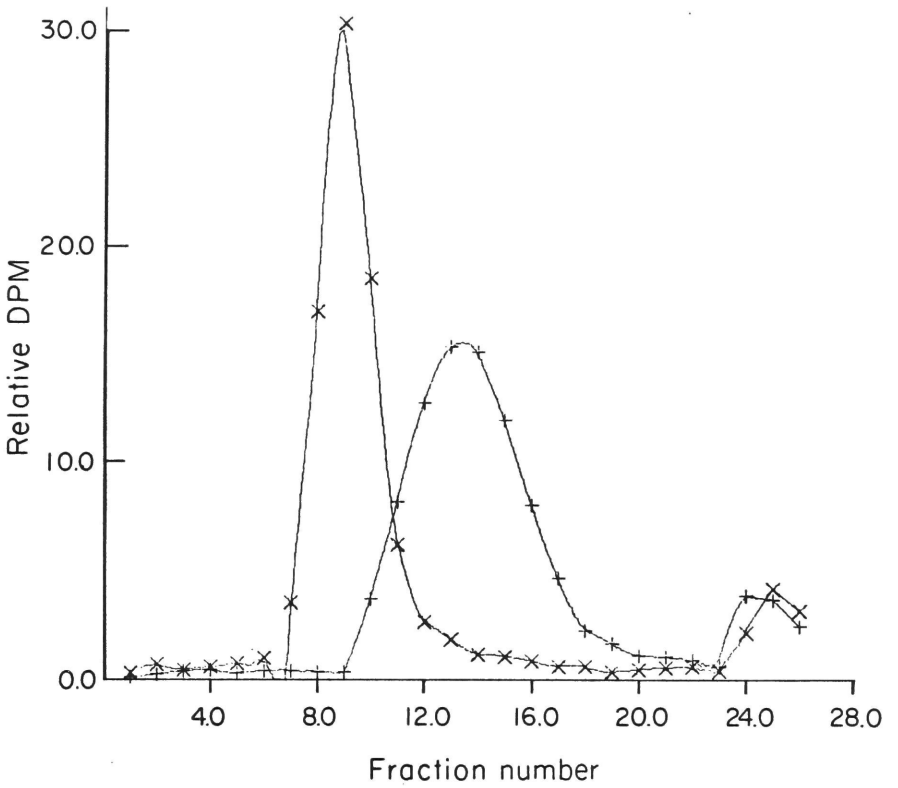

Figure A6: Graph made by Scintillation Data Program. This is an example of an interpolated relative plot of the data. There is a clear resolution of the marker and the experimental data.

x = ^{14}C marker DNA + = ^{3}H experimental DNA

COMPUTER PROGRAM 5

```
*     VELOCITY SEDIMENTATION- B. BURLINGHAM-  502 FOUNDERS
      DIMENSION T(100), EXPT(100), REXPT(100), S(100), DREXPT(100),
     1DTIME(100)
C     SENSE SWITCH 1-  OFF= DIRECT UV, ON= MONOCHROMETER
      PAUSE  1
      IF (SENSE SWITCH 1) 2, 3
    3 OMF= 0.1087
      GO TO 4
    2 OMF= 0.1153
      READ 41, ETAT, ETA20, ETAM, ETAW, NUBAR, RHOW20, RHOM
   41 FORMAT (7F10.5)
    4 READ 5, SAMPLE, OMEGA, N
    5 FORMAT (4X, 1A6, F10.4, I2)
   20 READ 6, (T(I), EXPT(I), I= 1, N)
    6 FORMAT (2F10.4)
      OMEGA= OMEGA*6.28318/60.0000
      NN= N - 1
      DO 7, I= 1, NN
      REXPT(I)= 7.3000 - OMF*EXPT(I)
      DREXPT(I)= LOGF(REXPT(I+1))-LOGF(REXPT(I))
      DTIME(I)= T(I + 1) - T(I)
    7 S(I)=  1.0000/OMEGA**2.000 *(DREXPT(I)/DTIME(I))
      SUMS= 0.00
      DO 8, I= 1, NN
    8 SUMS= SUMS + S(I)
      AVES= SUMS/ NN*1.000
      SUMDEV= 0.0000
      DO 9, I= 1, NN
    9 SUMDEV= (S(I) - AVES)**2.00
      SIGMA= SQRTF(SUMDEV/(NN*1.000))
      HIGHS= AVES + 2.000*SIGMA
      LOWS= AVES - 2.000*SIGMA
      DO 10, I= 1, NN
      IF (S(I) - LOWS) 11, 11, 12
   12 IF (S(I) - HIGHS) 10, 10, 11
   11 S(I)= 0.00
   10 CONTINUE
      KOUNT= 0
      SUMS= 00.00
      DO 13, I= 1, NN
      IF (S(I)) 13, 13, 14
   14 SUMS= SUMS + S(I)
      KOUNT= KOUNT + 1
   13 CONTINUE
      FAVES= SUMS/ (KOUNT*1000)
      KOUNT= 0
      SUMDEV= 0.00
      DO 15, I= 1, NN
      IF (S(I))   15, 15, 16
   16 SUMDEV= (S(I) -FAVES)**2.00
      KOUNT= KOUNT + 1
   15 CONTINUE
      FSIGMA= SQRTF(SUMDEV/(KOUNT*1.0000))
      S20W= ETAT/ETA20*ETAM/ETAW*(1.000-NUBAR*RHOW20)/(1.000-NUBAR*RHOM)
     1*FAVES
      CSIGMA= ETAT/ETA20*ETAM/ETAM*(1.000-NUBAR*RHOW20)/(1.000-NUBAR*RHO
     1M)*FSIGMA
      MWT= EXPF(0.35*LOGF(S20W/0.080))
      PRINT 17, SAMPLE, S20W, CSIGMA, FAVES, FSIGMA, KOUNT, AVES, SIGMA,
     1NN, MWT
   17 FORMAT  (1H0, 4X, 1A6, F10.3, F9.5, 2(10X, 2F10.3, 5X, I5),E9.3)
      GO TO 4
```

```
    END

 MEMORY MAP

 INTEGER VARIABLES
   IDENT    LEVEL    OBJECT CODE LOCATION   UP SUBROUTINE

   MWT        1            3 4177          NOT USED
   KOUNT      1            3 4221          NOT USED
   LOWS       1            3 4223          NOT USED
   NN         1            3 4260          NOT USED
   I          1            3 4274          1 2352
   N          1            3 4310          NOT USED
   NUBAR      1            3 4332          NOT USED
   IO         0            3 7775          1 2360

 FLOATING  POINT VARIABLES
   IDENT    LEVEL    OBJECT CODE LOCATION

   CSIGMA     1            3 4201
   S20W       1            3 4204
   FSIGMA     1            3 4207
   FAVES      1            3 4214
   HIGHS      1            3 4225
   SIGMA      1            3 4230
   SUMDEV     1            3 4233
   AVES       1            3 4236
   SUMS       1            3 4244
   OMEGA      1            3 4312
   SAMPLE     1            3 4315
   RHOM       1            3 4324
   RHOW20     1            3 4327
   ETAW       1            3 4334
   ETAM       1            3 4337
   ETA20      1            3 4342
   ETAT       1            3 4345
   OMF        1            3 4356

 FLOATING  POINT ARRAYS
   IDENT    LEVEL    OBJECT CODE LOCATION  DIMENSION   DIM1        DIM2

   DTIME      1            3 4361             1
   DREXPT     1            3 5035             1
   S          1            3 5511             1
   REXPT      1            3 6165             1
   EXPT       1            3 6641             1
   T          1            3 7315             1

 CONSTANTS
       VALUE          OBJECT CODE LOCATION

   0.80000000 E-01        3 4171
   0.35000000 E 00        3 4174
        1.000             3 4212
            0             3 4217
   0.0                    3 4241
   0.20000000 E 01        3 4247
   0.10000000 E 01        3 4252
   0.73000000 E 01        3 4255
   0.60000000 E 02        3 4262
```

```
     0.62831800 E 01          3 4265
     0.11530000 E 00          3 4350
     0.10870000 E 00          3 4353
              100             3 7771
                1             3 7773

 STATEMENT NUMBERS
    IDENT    LEVEL    OBJECT CODE LOCATION

    16           1            1 2130
    15           1            1 2155
    14           1            1 2026
    13           1            1 2046
    12           1            1 1734
    11           1            1 1747
    10           1            1 1755
    9            1            1 1622
    8            1            1 1547
    7            1            1 1474
    20           1            1 1335
    4            1            1 1321
    3            1            1 1263
    2            1            1 1271
    0            1            1 1256

 FORMAT  STATEMENTS
    IDENT    LEVEL    OBJECT CODE LOCATION

    17           1            3 4135
    6            1            3 4270
    5            1            3 4276
    41           1            3 4320

 LIBRARY FUNCTIONS
    IDENT    LEVEL    OBJECT CODE LOCATION

    (16120                    0 7264
    (R405I                    0 7340
    INPUT                     1 0001
    A**B                      1 0465
    EXPF                      1 0531
    LOGF                      1 0751
    SQRTF                     1 1127


 ERASABLE STORAGE      1 2370 TO 3 4135
```

COMPUTER PROGRAM 6

```
*       ERROR CHECK-  B. BURLINGHAM, 502 FOUNDERS
        DIMENSION COPNAM(10), J1BTM(200), K1BTM(200),L1BTM(200), INGRID(20
       10),ITEST(200), SUSAN (200), M1BTM (200), N1BTM (200), NNBTM(200)
C       NUMCOP MAX = 200
        I= MDUMPF (3)
        PAUSE 1
        REWIND 2
        ISTOP = 0
      1 READ 100, (COPNAM(I), I= 1, 10), NUMCOP, ISTOP
    100 FORMAT (10A6, I3, 7X, I1)
        WRITE OUTPUT TAPE 2, 100, COPNAM, NUMCOP, ISTOP
        READ 110, ( SUSAN(I), INGRID(I), M1BTM(I), J1BTM(I), K1BTM(I),
       1L1BTM(I), N1BTM(I), NNBTM(I), I= 1, NUMCOP)
    110 FORMAT (F10.4, 2X, I8, 3X, A4, 5(6X, A4))
        DO 12 I=1,NUMCOP
        IF (SUSAN(I) - 1) 16, 16, 17
     17 ITEST(I)= 3
        L1BTM(I)= K1BTM(I)
        K1BTM(I)= J1BTM(I)
        J1BTM(I)= M1BTM(I)
        GO TO 15
     16 IF (INGRID(I) - 1) 13, 13, 14
     13 MMM= M1BTM(I)
B       TEST= MMM/20202020
        IF (TEST) 142, 141, 142
    141 ITEST(I) = 4
        J1BTM(I)= L1BTM(I)
        K1BTM(I)= N1BTM(I)
        L1BTM(I) = NNBTM(I)
        GO TO 15
    142 J1BTM(I)= K1BTM(I)
        K1BTM(I)= L1BTM(I)
        L1BTM(I)= N1BTM(I)
        ITEST(I)= 2
        GO TO 15
     14 ITEST(I)= 1
B    15 J1BTM(I) = J1BTM(I)*00007777
B       K1BTM(I) = K1BTM(I)*00007777
B       L1BTM(I)= L1BTM(I)*00007777
B       IF(J1BTM(I)/00002020)4,3,4
      3 J1BTM(I) = 3
C       1 = NO BLANKS          2 = ONE BLANK          3 = TWO BLANKS
        GO TO 7
B     4 J1BTM(I) = J1BTM(I)*00000077
B       IF(J1BTM(I)/00000020) 6,5,6
      5 J1BTM(I) = 2
        GO TO 7
      6 J1BTM(I) = 1
B     7 IF(K1BTM(I)/00002020) 9,8,9
      8 K1BTM(I) = 3
        GO TO 112
B     9 K1BTM(I) = K1BTM(I)*00000077
B       IF(K1BTM(I)/00000020) 11,10,11
     10 K1BTM(I) = 2
        GO TO 112
     11 K1BTM(I) = 1
B   112 IF (L1BTM(I)/00002020) 113, 114, 113
    114 L1BTM(I) = 3
        GO TO 12
B   113 L1BTM(I)= L1BTM(I)*00000077
B       IF (L1BTM(I)/00000020) 115, 116, 115
    116 L1BTM(I)= 2
```

```
      GO TO 12
  115 L1BTM(I)= 1
   12 CONTINUE
      WRITE OUTPUT TAPE 2,120, (ITEST(I), J1BTM(I), K1BTM(I), L1BTM(I),
     1 I= 1, NUMCOP)
  120 FORMAT(33X,I4,     3(6X, I4) )
      IF (ISTOP) 1, 1, 20
   20 ENDFILE 2
      REWIND 2
      STOP
      END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
MMM	1	3 0333	NOT USED
NUMCOP	1	3 0367	NOT USED
ISTOP	1	3 0373	NOT USED
I	1	3 0377	1 2466
IO	0	3 7775	1 2476

FLOATING POINT VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION
TEST	1	3 0330

INTEGER ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
NNBTM	1	3 0401	1		
N1BTM	1	3 1221	1		
M1BTM	1	3 2041	1		
ITEST	1	3 4011	1		
INGRID	1	3 4631	1		
L1BTM	1	3 5451	1		
K1BTM	1	3 6271	1		
J1BTM	1	3 7111	1		

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
SUSAN	1	3 2661	1		
COPNAM	1	3 7731	1		

CONSTANTS

VALUE	OBJECT CODE LOCATION
16	3 0312
63	3 0314
1,040	3 0316
0	3 0320
2	3 0322
4	3 0324
2,130,960	3 0326
0	3 0371
3	3 0375
200	3 7767
10	3 7771
1	3 7773

STATEMENT NUMBERS IDENT	LEVEL	OBJECT CODE LOCATION
20	1	1 2434
116	1	1 2325
115	1	1 2335
114	1	1 2272
113	1	1 2302

10	1	1 2241
11	1	1 2251
112	1	1 2257
8	1	1 2206
9	1	1 2216
5	1	1 2155
6	1	1 2165
7	1	1 2173
3	1	1 2122
4	1	1 2132
141	1	1 1765
142	1	1 2017
14	1	1 2051
13	1	1 1740
15	1	1 2057
17	1	1 1673
16	1	1 1725
12	1	1 2343
1	1	1 1437
0	1	1 1415

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION
120	1	3 0276
110	1	3 0335
100	1	3 0356

LIBRARY FUNCTIONS

IDENT	LEVEL	OBJECT CODE LOCATION
(R405I		0 7376
(R162I		0 7462
(W1620		1 0001
INPUT		1 0163
MDUMPF		1 0647

ERASABLE STORAGE 1 2506 TO 3 0276

COMPUTER PROGRAM 7

```
*     ERROR CORRECT-  B. BURLINGHAM, 502 FOUNDERS
      DIMENSION ORGNAM(10),COPNAM(10),J1(200),J1BTM(200),K1(200),K1BTM(2
     100),ESR(200),T(200), C1(200), C2(200),INGRID(200), L1(200), L1BTM(
     2200), C3(200)
  100 FORMAT (10A6, I3, 7X, I1)
  101 FORMAT ( 1X, 10A6, I3, 7X, I1)
  102 FORMAT (8F10.5)
  110 FORMAT (5X, I3, 2X, 10A6, I3, 7X, I1)
  130 FORMAT(14HCHECK IF WRONG)
  140 FORMAT(F10.4,10X,F9.4,3(I8,2X))
  150 FORMAT(10X, F10.4, 10X, F9.4, 3(I8, 2X))
  151 FORMAT (10X, F9.4, 3(I8, 2X))
  120 FORMAT(36X,I1,     3(9X, I1)  )
      I= MDUMPF (3)
C     CORRECTED DATA ON TAPE DECK 7
C     ARRAYS FOR CONTROLLING CORRECTIONS ON TAPE DECK 5
      PAUSE 2
C     SENSE SWITCH 1 ON= THREE CHANNEL  OFF= TWO CHANNEL
C     SENSE SWITCH 2  ON= ESR   OFF= CHANNELS ONLY
C     SENSE SWITCH 4 ON = CHROMATOGRAPHY FORMAT CALL CARD
      IF (SENSE SWITCH 4) 1031, 1030
 1031 READ 1032, N, NN
 1032  FORMAT (I3, I1)
      GO TO 1033
 1030  READ 103, N, NN
  103   FORMAT (I5, I2)
 1033 NUMBER= 1
      ISTOP=0
C     REWIND 7/ I=XFILEF(7, N)
      REWIND 5
      REWIND 7
      I=XFILEF (7, N)
      PRINT 14, N, NN
   14  FORMAT (1H1, 1X, 18HBEGINNING- I=FILEF,I3, 6H, TAPE, I2)
    1 READ 100, (ORGNAM(I), I= 1, 10), NUMORG, ISTOP
      READ INPUT TAPE 5, 100,(COPNAM(I), I= 1,10), NUMCOP, ISTOP
      IF(NUMORG-NUMCOP) 2,3,2
    2 WRITE TYPE 130
    3 WRITE OUTPUT TAPE 7,100,(ORGNAM(I), I= 1, 10), NUMORG, ISTOP
      READ INPUT TAPE 5,120,(INGRID(I), J1BTM(I), K1BTM(I), L1BTM(I),
     1 I= 1, NUMORG)
      DO 91, I= 1, NUMORG
      KK= INGRID(I)
      GO TO (12,13, 16, 17) KK
   17 READ 153, ESR(I), T(I), J1(I), K1(I), L1(I)
  153 FORMAT (20X, F10.4, 10X, F9.4, 3(I8, 2X))
      GO TO 15
   16 READ 151, T(I), J1(I), K1(I), L1(I)
      GO TO 15
   13 READ 150,  ESR(I), T(I), J1(I), K1(I), L1(I)
      GO TO 15
   12 READ 140,  ESR(I), T(I), J1(I), K1(I), L1(I)
   15 JJ = J1BTM(I)
      GO TO (6,5,4) JJ
    4 J1(I) = J1(I)/10
    5 J1(I) = J1(I)/10
    6 KK = K1BTM(I)
      GO TO (9,8,7) KK
    7 K1(I) = K1(I)/10
    8 K1(I) = K1(I)/10
    9 LL= L1BTM(I)
      GO TO (91, 92, 93)LL
```

```
   93 L1(I)= L1(I)/10
   92 L1(I)= L1(I)/10
   91 CONTINUE
      DO33, I= 1, NUMORG
      IF (T(I)) 112, 112, 111
  112 C1(I)= 0.0
      C2(I)= 0.0
      C3(I)= 0.0
      GO TO 33
  111 C1(I)=J1(I)*1.0/T(I)
      C2(I)=K1(I)*1.0/T(I)
      C3(I)= L1(I)*1.0/T(I)
   33 CONTINUE
      PRINT 110, NUMBER, (ORGNAM(I), I= 1, 10), NUMORG, ISTOP
      IF (SENSE SWITCH 2) 37, 38
   38 IF (SENSE SWITCH 1) 35, 34
   34 WRITE OUTPUT TAPE 7, 500, (C1(I), C2(I), I=1, NUMORG)
  500 FORMAT (2F12.8)
      GO TO 36
   35 WRITE OUTPUT TAPE 7, 501,(C1(I), C2(I), C3(I), I= 1, NUMORG)
  501 FORMAT (3F12.8)
      GO TO 36
   37 WRITE OUTPUT TAPE 7, 502,(ESR(I), C1(I), C2(I), C3(I),I=1,NUMORG)
  502 FORMAT (4F12.4)
   36 NUMBER= NUMBER+ 1
      IF (ISTOP) 1, 1, 18
   18 ENDFILE 7
      REWIND 7
      REWIND 5
      STOP
      END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
LL	1	2 4137	NOT USED
JJ	1	2 4141	NOT USED
KK	1	2 4164	NOT USED
NUMCOP	1	2 4166	NOT USED
NUMORG	1	2 4170	NOT USED
ISTOP	1	2 4227	NOT USED
NUMBER	1	2 4231	NOT USED
NN	1	2 4243	NOT USED
N	1	2 4245	NOT USED
I	1	2 4251	1 3751
IO	0	3 7775	1 3761

INTEGER ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
L1BTM	1	2 5553	1		
L1	1	2 6373	1		
INGRID	1	2 7213	1		
K1BTM	1	3 4573	1		
K1	1	3 5413	1		
J1BTM	1	3 6233	1		
J1	1	3 7053	1		

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2

C3	1	2 4423	1
C2	1	3 0033	1
C1	1	3 1163	1
T	1	3 2313	1
ESR	1	3 3443	1
COPNAM	1	3 7673	1
ORGNAM	1	3 7731	1

CONSTANTS

VALUE	OBJECT CODE LOCATION
0.10000000 E 01	2 4131
0.0	2 4134
7	2 4223
0	2 4225
3	2 4247
200	3 7767
10	3 7771
1	3 7773

STATEMENT NUMBERS

IDENT	LEVEL	OBJECT CODE LOCATION
18	1	1 3713
36	1	1 3674
34	1	1 3506
35	1	1 3552

38	1	1 3502
37	1	1 3622
111	1	1 3356
112	1	1 3332
33	1	1 3422
93	1	1 3253
92	1	1 3263
7	1	1 3212
8	1	1 3222
9	1	1 3232
4	1	1 3151
5	1	1 3161
6	1	1 3171
15	1	1 3130
17	1	1 2766
16	1	1 3020
13	1	1 3046
12	1	1 3100
91	1	1 3273
3	1	1 2611
2	1	1 2605
1	1	1 2460
1033	1	1 2411
1030	1	1 2375
1031	1	1 2357
0	1	1 2343

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION
502	1	2 4115
501	1	2 4121
500	1	2 4125
153	1	2 4143
14	1	2 4172
103	1	2 4233
1032	1	2 4237
120	1	2 4253
151	1	2 4270
150	1	2 4303
140	1	2 4324
130	1	2 4341
110	1	2 4353
102	1	2 4372
101	1	2 4376
100	1	2 4412

LIBRARY FUNCTIONS

IDENT	LEVEL	OBJECT CODE LOCATION
(16120		0 7264
(R405I		0 7340
(RWTF		1 0001
(R162I		1 0453
(W1620		1 0647
INPUT		1 1031
MDUMPF		1 1515
XFILEF		1 2263

ERASABLE STORAGE 1 3773 TO 2 4115

COMPUTER PROGRAM 8

```
*     SCINTILLATION DATA- BYRON BURLINGHAM- 502 FOUNDERS- 30 SEPT., 1967
      DIMENSION H( 40),C( 40),ZH( 40),ZC( 40),  C2(40),       QH( 40),QC
     1( 40),DH( 40),DC( 40),W( 40),X(40),Z(10),SDH( 40),SDC( 40),U(10),
     2AREAH(10),          DW(400), DDW(400),       DX( 400), XAXH(10),
     3MAXC(10),          S20W(10),C1(400), XW(10), MAX(10),
     4          BAD(4, 5) ,  GDH(8), AI(40), GL(8)
C     S20W= AD2 31.1/34.1   AD12 30.6/ 33.5        VIRUS = 800 S
      EQUIVALENCE (DX, C),(DX, C1),     (DW,H), (ZH, W),(ZC,X),(QH,GL),
     1(QH, AI),                (QH, S20W),            (QC, XW), (DH, C2),
     2              (SDH, BAD), (SDC, MAXC),(SDC, AREAH),(QC,GDH),
     3 (DC, XAXH), (DX, DDW), (SDH, MAX)
      I= MDUMPF (3)
   92 PAUSE 1
C      INPUT TAPE ON 7, HIGH DENSITY
      READ 91, NUMB, NNUMB, XMARK, KOOK
   91 FORMAT (I5, I2, F6.2, I1)
      REWIND 7
      I= XFILEF (7, NUMB)
      READ 110,(U(I), I=1,10)
  110 FORMAT(10F6.3)
      READ 111, A, B, HC, CC, BGH, BGC, EH, EC
  111 FORMAT (8F10.5)
      NUMBER = 0
   22 PAUSE 22
C     REMINDER-- SET ORIGIN
C     PAUSE 22- DATA INPUT
C     DIRECT PLOT = 1 ON,  XTH ORDER PLOT= 1 OFF
C      LOG PLOT-- 4 ON, RELATIVE PLOT-- 4 OFF
C     MOLECULAR WEIGHT DETERMINATION = 2 OFF
C     KOOK= 1 NEUTRAL DNA, KOOK= 2, ALKALINE DNA, KOOK= 3 GLOBULAR PARTICLE
    1 READ INPUT TAPE 7, 112, (Z(I), I= 1, 10), N, ISTOP
  112 FORMAT(10A6, T3, 7X, I1)
      NUMBER = NUMBER + 1
       IF (SENSE SWITCH 4) 23, 24
   24  Q=PLOTF (40.00, 5.0, 1)
      XP1=-0.600
      XP2=-1.20
      XP3=-2.000
      XP4= 0.30
       GO TO 25
   23 Q=PLOTF (40.0,.5000,1)
      XP1= -0.075
      XP2= -0.15
      XP3= -0.25
      XP4= 0.031
   25 Q=PLOTF (0.0,0.0,2)
       N1=N-2
      N2= (N-1)
      N3= N-3
      N4= 10*N
      N5= N1*10
      N6= N2*10
      N7= N + 1
      READ INPUT TAPE 7, 2, (H(I), C(I), I= 1, N)
    2 FORMAT (2F12.3)
      DO 3 I=1,N
      ZH(I)=H(I)-BGH
    3 ZC(I)=C(I)-BGC
      HCF= (HC- 1.000)/N
      CCF= (CC - 1.000)/N
      DO 5 I=1,N
      QH(I)=       (HC-I*HCF) * ((B*ZH(I) - ZC(I))/(B-A))
```

```
    5 QC(I)=(CC-I*CCF)*(B*(ZC(I)-(A*ZH(I)))/(B-A))
      DO 7 I=1,N
    7 DC(I)=QC(I)/EC
      DH(I)=QH(I)/EH
      DO 88 I=1,N
      SDH(I)= SQRTF(DH(I))
   88 SDC(I)= SQRTF(DC(I))
      PRINT 8, (Z(I), I= 1, 10), NNUMB, NUMB, NUMBER
    8 FORMAT (1H1, 1X,10A6, 30X, I6, I6, I6)
      PRINT 9
    9 FORMAT (1X, 6HSAMPLE, 7X, 3HRED, 6X,  6HH3--BC, 5X, 6HH3--QC, 5X,
     16HH3-DPM, 5X, 6H3SD-H3, 5X, 5HGREEN, 6X, 6HC14-BC, 5X, 6HC14-QC,
     25X, 7HC14-DPM, 4X, 7H3SD-C14, /)
      PRINT 10, (I, H(I), ZH(I), QH(I), DH(I),  SDH(I), C(I), ZC(I),
     1QC(I), DC(I),  SDC(I), I=1,N)
   10 FORMAT (2X, I3, 4X, F9.1, 2X, F9.1, 2X, F9.1,2X, F9.1, 4X, F6.1,
     13X, F9.1, 2X,F9.1, 2X, F9.1, 2X, F9.1, 4X, F6.1)
      RAREA= 1.000
      TAREA= 1.000
       DO 13, I= 1, N
       IF (DH(I) - 1) 12, 12, 11
   12  DH(I)= 0.0
       W(I)= 0.0
       GO TO 14
   11 W(I)= LOGF(DH(I))/2.303
       TAREA= TAREA + DH(I)
      IF (W(I)) 12, 14, 14
   14 IF (DC(I)-1.0) 26, 26, 15
   26  DC(I)= 0.0
       X(I)= 0.0
       GO TO 13
   15  X(I)= LOGF(DC(I))/2.303
       RAREA= RAREA + DC(I)
      IF (X(I)) 26, 13, 13
   13  CONTINUE
       IF (SENSE SWITCH 4) 29, 16
   16   DO 28, I= 1, N
       W(I)= (DH(I)*100.0)/TAREA
   28  X(I)= (DC(I)*100.0)/RAREA
   29 Q=PLOTF (10.0,W(1), 3)
      IF (SENSE SWITCH 1) 117, 400
  400 KROW= 4
      KCOL= 5
      L= 10
      C1(10)= DH(1)
      DH(N7)= DH(N)
      DO 470, J= 1, N1
      KONT= 2
      J2= J + 1
      J4= J +  3
      J5= J + 2
      GL(2)= (J2*10.000)
      GL(3)= (J5*10.000)
      GDH(2)= DH(J2)
      GDH(3)= DH(J5)
      DO 470, MH= 1,2
      KONT= KONT - 1
      IF (KONT) 37, 37, 38
   37 GL(4)= J4*10.000
      GDH(4)= DH(J4)
   38 DO 470, M1= 1,5
      GDH(1)= C1(L)
```

```
      GL(1)= L*1.000
      DO 471, I= 1, KROW
      DO 471, K= 1, KCOL
  471 BAD(I,K)= 0.0
      DO 473, M= 1,4
      XV= GL(M)
      DO 473, K= 1, KROW
      XT= (1.0/XV**2)*XV**K
      BAD(K,KCOL)= BAD(K,KCOL) +GDH(M)*XT*XV
      DO 473, I= 1, KROW
  473 BAD(I,K) = BAD(I,K) + XT*XV**I
      CALL SOLVE ( BAD, KROW, KCOL)
      L = L + 1
      ZEL= L*1.000
      C1(L)= BAD(1, KCOL) + BAD(2, KCOL)*ZEL + BAD(3,KCOL)*ZEL**2  +
     1BAD(4,KCOL)*ZEL**3
      CALL CHECK (DH, J, J2, C1, L)
  470 CONTINUE
  177 DO 178 I= 1, N
      Q=PLOTF (AI, (W(I)-XP4 ),  3)
      Q=PLOTF (AI, (W(I)-XP4  ), 4)
      Q=PLOTF (AI, (W(I)+XP4  ), 4)
      Q=PLOTF (AI, (W(I)-XP4  ), 4)
      Q=PLOTF (AI, (W(I)-XP4  ), 3)
      Q=PLOTF ((AI+2.5),  W(I), 3)
      Q=PLOTF ((AI+2.5),  W(I), 4)
      Q=PLOTF ((AI-2.5),  W(I), 4)
      Q=PLOTF ((AI+2.5),  W(I), 4)
  178 Q=PLOTF ((AI+2.5),  W(I), 3)
      IF (SENSE SWITCH 4) 409, 410
  409 DO 1280, I= 10, N4
      IF(C1(I)-1)1279, 1279,  1785
 1279 DW(I)= 0
      GO TO 1280
 1785 DW(I)= LOGF(C1(I))/2.303
 1280 CONTINUE
      GO TO 420
  410 DO 411, I= 10, N4
       IF ( C1(I) - 1 ) 412, 412, 413
  412 DW(I)= 0.0
      GO TO 411
  413  DW(I)= (C1(I)*100.0)/TAREA
  411  CONTINUE
  420 Q=PLOTF (10.0, W(1), 3)
      DO 1378, I= 10, N5
      AI=I
 1378 Q= PLOTF (AI,DW(I), 4)
      AI= N6
      Q=PLOTF (AI, W(N2), 4)
      AI= N4
      Q= PLOTF (AI, W(N), 4)
      AI= 10*I
      Q= PLOTF (AI, W(N), 3)
      GO TO 179
  117 DO 17  I=1,N
      AI= 10*I
   17 Q=PLOTF (AI, W(I), 4)
      Q= PLOTF     (N,  W(N), 3)
  179 Q=PLOTF (10., X(1), 3)
      IF (SENSE SWITCH 1)  118, 500
  118 PAUSE 33
C     CHANGE PEN
```

```
      DO 18  I=1,N
      AI= 10*I
   18 Q=PLOTF (AI, X(I), 4)
      Q= PLOTF     (N,  X(N), 3)
      GO TO 190
  500 L= 10
      C1(10)= DC(1)
      DC(N7)= DC(N)
      DO 570, J= 1, N1
      KONT= 2
      J2= J + 1
      J3= J + 3
      J5= J + 2
      GL(2)= (J2*10.000)
      GL(3)= (J5*10.000)
      GDH(2)= DC(J2)
      GDH(3)= DC(J5)
      DO 570, MH= 1,2
      KONT= KONT - 1
      IF (KONT) 47, 47, 48
   47 GL(4)= J3*10.000
      GDH(4)= DC(J3)
   48 DO570, M1= 1, 5
      GDH(1)= C1(L)
      GL(1)= L*1.000
      DO 571,  I= 1,KROW
      DO 571, K= 1,KCOL
  571 BAD(I,K)= 0.00
      DO 573, M= 1,4
      XV= GL(M)
      DO 573, K= 1,KROW
      XT= (1.00/XV**2)*XV**K
      BAD(K,KCOL)= BAD(K,KCOL) +GDH(M)*XT*XV
      DO 573, I= 1,KROW
  573 BAD(I,K)= BAD(I,K) + XT*XV**I
      CALL SOLVE ( BAD, KROW, KCOL)
      L= L + 1
      ZEL= L*1.000
      C1(L) = BAD(1,KCOL) + BAD(2, KCOL)*ZEL + BAD(3, KCOL)*ZEL**2 + BAD
     1(4, KCOL)*ZEL**3
      CALL CHECK (DC, J, J2, C1, L)
  570 CONTINUE
  188 DO 189 I= 1, N
      AI= 10*I
      Q=PLOTF ((AI + 2.5), (X(I) - XP4  ), 3)
      Q=PLOTF ((AI + 2.5), (X(I) - XP4  ), 4)
      Q=PLOTF ((AI - 2.5), (X(I) + XP4  ), 4)
      Q=PLOTF ((AI + 2.5), (X(I) - XP4  ), 4)
      Q=PLOTF ((AI - 2.5), (X(I) + XP4  ), 4)
      Q=PLOTF ((AI + 2.5), (X(I) - XP4  ), 3)
      Q=PLOTF ((AI - 2.5), (X(I) - XP4  ), 3)
      Q=PLOTF ((AI - 2.5), (X(I) - XP4  ), 4)
      Q=PLOTF ((AI + 2.5), (X(I) + XP4  ), 4)
      Q=PLOTF ((AI - 2.5), (X(I) - XP4  ), 4)
  189 Q=PLOTF ((AI - 2.5), (X(I) - XP4  ), 3)
      IF (SENSE SWITCH 4) 421, 422
  421 DO 1884 I= 10, N4
      IF(C1(I)-1)1883, 1883, 1882
 1883  DX(I) = 0.0
      GO TO 1884
 1882 DX(I)= LOGF(C1(I))/2.303
 1884  CONTINUE
```

```
      GO TO 423
  422 DO 424, I= 10, N4
      IF (C1(I) - 1 ) 425, 425, 426
  425 DX(I)= 0.0
      GO TO 424
  426 DX(I)= (C1(I)*100.0)/RAREA
  424   CONTINUE
  423 Q= PLOTF (10.0, X(1), 3)
      DO 1388, I= 10, N5
      AI= I
 1388 Q=PLOTF (AI, DX(I), 4)
      AI= N6
      Q=PLOTF (AI, X(N2), 4)
      AI= N4
      Q= PLOTF (AI, X(N), 4)
      Q= PLOTF (AI, X(N), 3)
  190 Q=PLOTF (0.0, 0.0, 3)
      Q=PLOTF (0.0, 0.0, 4)
      DO 19 IS= 40, 360, 40
      S= IS
      Q=PLOTF  (S, 0, 4)
      Q=PLOTF (S, XP1,    4)
      SZ= (S-10.)
      Q=PLOTF (SZ,  XP2,         3)
      RZ= IS*0.1
      PRINT (PLOTTA) 119, RZ
  119 FORMAT (3H  1, F5.1)
      Q=PLOTF (S, XP1,    3)
   19 Q=PLOTF (S, 0.0, 4)
      Q=PLOTF (0.0, 0.0, 4)
      Q=PLOTF (150., XP3,  3)
      PRINT (PLOTTA) 1119
 1119 FORMAT (3H  1, 15HFRACTION NUMBER)
      Q= PLOTF ( 0, 0, 3)
       IF (SENSE SWITCH 4) 30, 31
   30 DO 20 I= 1,10
      Q=PLOTF (0.0, U(I), 4)
      Q=PLOTF (-7.5, U(I), 4)
   20 Q=PLOTF (0.0, U(I), 4)
      Q=PLOTF (0.0, 0.0, 4)
      Q=PLOTF (0.0, 0.0, 3)
      Q= PLOTF (-15.,1.5, 3)
      PRINT (PLOTTA) 2002
 2002 FORMAT (3H2 1, 3HDPM)
      DO 2004, I= 1,10
      Q= PLOTF (-30.0, U(I), 3)
      R= EXPF(U(I)*2.303)
 2004 PRINT (PLOTTA) 2003, R
 2003 FORMAT (3H  1, F8.0)
      Q= PLOTF (10.,5.0, 3)
      PRINT (PLOTTA) 2000, (Z(I), I= 1, 5)
 2000 FORMAT (3H  1, 5A6)
      Q=PLOTF (20.,4.9, 3)
      PRINT (PLOTTA) 2000, (Z(I), I= 6,10)
      Q=PLOTF (0.0, 6.0, 3)
      Q=PLOTF (0.0, 0.0, 2)
      GO TO 53
   31  DO 50, I= 10, 50, 10
       RZ= I*1.00
      Q=PLOTF (0.0, RZ, 4)
       Q=PLOTF ( -7.5, RZ, 4)
       Q=PLOTF ( -7.5, RZ, 3)
```

```
      Q=PLOTF ( -20.0, RZ, 3)
      PRINT (PLOTTA) 51, RZ
   51 FORMAT (3H  1, F5.1)
      Q=PLOTF ( -7.5, RZ, 3)
   50 Q=PLOTF (0.0, RZ, 4)
      Q=PLOTF (0.0, RZ, 3)
      Q=PLOTF(-20.0, 20.0, 3)
      PRINT (PLOTTA) 52
   52 FORMAT (3H2 1, 12HRELATIVE DPM)
      Q=PLOTF ( 10.0, 50.0, 3)
      PRINT(PLOTTA) 2000, (Z(I), I= 1, 5)
      Q=PLOTF ( 20.0, 49.0, 3)
      PRINT (PLOTTA) 2000,  (Z(I), I= 6, 10)
      Q=PLOTF (0.0, 60.0, 3)
      Q=PLOTF (0.0, 0.0, 2)
   53 IF (SENSE SWITCH 2)  22, 201
  201 IF (SENSE SWITCH 1) 2005, 2006
 2005 DO 2007, I= 1, N
      L= I*10
      DX(L)= X(I)
 2007 DW(L)= W(I)
      N5= 10
      N6= 20
      GO TO 301
 2006 N5=1
      N6= 2
  301 DO 3010 J= 1, 5
 3010 MAXC(J) = 0
      CALL MAXY(DX, N4, N5, N6, MAX)
      MC= 0
      DX(1)= 0.0
      DO 315, J= 1, 10
      MAXC(J)= MAX(J)
      IF (MAXC(J))  315, 315, 3150
 3150 MC= MC + 1
  315 CONTINUE
      IF (MC - 1) 317, 3170, 318
  317 XMAX= 14.1
      GO TO 204
 3170 XMAX= MAX(1)/10.00
      GO TO 204
  318 DO 320, J= 1, MC
      IT= MAXC(J)
      DO 320,  I= 1, MC
      IF (J-I) 319, 320, 319
  319 ID= MAXC(I)
      PMAX= DX(IT) - DX(ID)
      IF ( PMAX) 316, 316, 320
  316 MAXC(J)= 1
  320 CONTINUE
      DO 322, J= 1, MC
      IF (MAXC(J) - 1) 322, 322, 321
  321 XMAX= MAXC(J)/10.00
  322 CONTINUE
  204 DO 2010 J= 1, 10
 2010 XAXH(J)= 0
      CALL MAXY(DW, N4, N5, N6, MAX)
      MH= 0
      DO 215 J= 1, 10
      IF (MAX(J)) 215, 215, 21
   21 XAXH(J)= MAX(J)/10.000
      MH = MH + 1
```

```
  215 CONTINUE
 2151 IF (MH-1)  2152, 2166, 2166
 2152 PRINT 2155
 2155 FORMAT(1H0,  23HNO EXPERIMENTAL MAXIMUM)
      GO TO 601
 2166 DO 216 J= 1, MH
  216 S20W(J)= XMARK*((N-1.5) - XAXH(J))/ ((N-1.5) - XMAX)
      GO TO(21706,21704, 21705), KOOK
21706 DO 217 J= 1, MH
  217 XW(J)= EXPF((LOGF(S20W(J)) - LOGF(0.0882))/0.346)
      XMW=  EXPF((LOGF(XMARK) - LOGF(0.0882))/0.346)
      GO TO 21703
21704  DO 21701, J= 1, MH
21701 XW(J)= EXPF((LOGF(S20W(J)) - LOGF(0.0528))/ 0.400)
      XMW= EXPF((LOGF(XMARK)- LOGF(0.0528))/0.400)
      GO TO 21703
21705 DO 21702, J= 1, MH
21702 XW(J)=(SQRTF(S20W(J)**3))/.0001131328
      XMW= (SQRTF(XMARK ** 3))/ .0001131328
21703 IF (SENSE SWITCH 4) 72, 73
   73  TAREA= 0.0
      SZ= N5/10.000
       DO 74, I= 10, N4, N5
   74 TAREA= TAREA + DW(I)*SZ
   72 KCOL= 4
      KROW= 3
      DO 2170 J= 1, MH
      J2= (XAXH(J)*10 - 20)
      J5= (XAXH(J)*10 + 20)
      DO 2171 I= 1, 3
      DO 2171, K= 1, 4
 2171 BAD(I, K) = 0.0
      IF (SENSE SWITCH 4) 75, 76
   75 DO 2173, M= J2, J5, N5
      BAD(1,4)= BAD(1,4) + EXPF(DW(M)*2.303)
      BAD (2,4) = BAD(2,4) + EXPF(DW(M)*2.303)*M
      BAD(3,4)= BAD(3,4) + EXPF(DW(M)*2.303)*M**2
       GO TO 77
   76 DO 2173, M= J2, J5, N5
      BAD(1,4)= BAD(1,4) + DW(M)
       BAD(2,4)= BAD(2,4) + DW(M)*M
       BAD(3,4) = BAD(3,4) + DW(M)*M**2
   77 XV=M
      DO 2173, K=1,3
      XT= (1.0/XV**2)*XV**K
      DO 2173, I= 1,3
 2173 BAD(I,K)= BAD(I,K) + XT*XV**I
      CALL SOLVE ( BAD, KROW, KCOL)
      K= 1
      M= 2
      XAXH(J)= XAXH(J)*10.00
      DO 2174, I= 1, M
      DDW(I)= (BAD(1,4))+ (XAXH(J) + 1.0 - I)*(BAD(2,4)) +
     2(XAXH(J) + 1.0 - I)**2*(BAD(3,4))
      K= K + 1
      IF (DDW(I)) 2175, 2176, 2176
 2175 DDW(I)= 0.0
      GO TO 2177
 2176 M= M + 1
 2174  CONTINUE
 2177 M1= 2
      DO 2178, I= 1, M1
```

```
      DDW(K)= (BAD(1,4))+(XAXH(J)+I)*(BAD(2,4))+(XAXH(J)+I)**2* BAD(3,4)
      K=K+1
      IF (DDW(K)) 2179, 21791, 21791
 2179 DDW(K)= 0
      GO TO 21792
21791 M1= M1+1
 2178 CONTINUE
21792 AREAH(J) = 0.0
      K= K-1
      DO 21781, I= 1, K
21781 AREAH(J) = AREAH(J) + DDW(I)
   66 AREAH(J)= 100.00 *AREAH(J)/TAREA
   67 XAXH(J)= XAXH(J)/10.00
 2170  CONTINUE
      PRINT 218,
  218 FORMAT(1H0,11X,14HMARKER MAXIMUM,10X,4HS20W, 10X, 23HMARKER MOLECU
     1LAR WEIGHT,  // )
      PRINT 219, XMAX, XMARK, XMW
  219 FORMAT (18X, F6.1, 9X,  F8.2,    14X, F11.0, //)
      PRINT 220,
  220 FORMAT (8X,  22HEXPERIMENTAL MAXIMUM, 6X, 4HS20W, 13X, 16HMOLECULA
     1R WEIGHT, 10X, 13HRELATIVE AREA , //)
      PRINT 221,(XAXH(I), S20W(I), XW(I), AREAH(I),  I=1, MH)
  221 FORMAT (18X, F5.1, 9X, F8.2, 14X, F11.0, 16X, F7.2 )
  601 IF (SENSE SWITCH 1) 22, 599
  599 IF(ISTOP) 1, 1, 600
  600 REWIND 7
      GO TO 92
      END
      SUBROUTINE SOLVE (BAD, KROW, KCOL)
       DIMENSION BAD(4,5)
      DO 21720, I= 1, KROW
      SAD = BAD( I, I)
      DO 21721,  J= 1, KCOL
21721 BAD( I, J)= BAD(I,J)/SAD
      DO 21720, J= 1, KROW
      IF (J-I) 21722, 21720, 21722
21722 CAD= BAD(J,I)
      DO 21720, M= 1, KCOL
      BAD(J, M)= BAD (J,M) -  BAD(I,M)*CAD
21720 CONTINUE
      RETURN
      END
      SUBROUTINE CHECK (DH, J, J2, C1, L)
      DIMENSION DH(40), C1(400)
      CORECT= ((DH(J) - DH(J2))/10.00)
      IF (DH(J) - DH(J2)) 477, 476, 476
  476 XCK= DH(J2)*0.90
      IF (C1(L) - XCK) 475, 475, 474
  474 XCK= DH(J)*1.25
      IF (C1(L) - XCK) 470, 475, 475
  475 C1(L)= DH(J) - CORECT*(L-J*10.00)
      GO TO 470
  477 XCK= DH(J) - DH(J)/10.00
      IF (C1(L) - XCK) 478, 478, 479
  479 XCK= DH(J2)*1.25
       IF (C1(L)-XCK) 470, 478, 478
  478 C1(L)= DH(J) + CORECT*(L - J*10.00)
  470 CONTINUE
      RETURN
      END
      SUBROUTINE MAXY(DW, N4, N5, N6, MAX)
```

```
      DIMENSION  DW(400), MAX(10)
      DO 203, J= 1, 10
  203 MAX(J)= 0
      J= 1
      DO 213 I= 30, N4, N5
      DO 213 I= 30, N4, N5
      DDW    = DW(I) - DW(N7)
  202 IF(DDW    )213, 205, 205
  205 IF (DW(I) -1.0)  213, 213, 2080
 2080 IF(DW(I) - 2.707) 208, 208, 211
  208 N8= I + N6
      D2DW   = DW(N7)    - DW(N8)
      IF(D2DW    ) 213, 211, 211
  211 N7= I - N5
      D3DW   = DW(I) - DW(N7)
      IF(D3DW    ) 213, 214, 214
  214 MAX(J)= I
      J= J + 1
  213 CONTINUE
      RETURN
      END
```

MEMORY MAP

INTEGER VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION	UP SUBROUTINE
N8	4	2 7736	2 7104
N7	4	2 7743	2 7112
I	4	2 7752	2 7120
J	4	2 7754	2 7126
M	2	2 7772	2 7156
J	2	2 7777	2 7166
I	2	3 0004	2 7200
ID	1	3 0256	2 7216
IT	1	3 0260	2 7224
MC	1	3 0270	NOT USED
IS	1	3 0456	NOT USED
J3	1	3 0460	2 7232
M	1	3 0476	2 7240
K	1	3 0500	2 7246
M1	1	3 0502	NOT USED
MH	1	3 0504	NOT USED
J5	1	3 0506	2 7260
J4	1	3 0510	2 7266
J2	1	3 0512	2 7274
KONT	1	3 0514	NOT USED
J	1	3 0516	2 7302
L	1	3 0520	2 7312
KCOL	1	3 0522	2 7320
KROW	1	3 0524	NOT USED
N7	1	3 1007	2 7330
N6	1	3 1011	NOT USED
N5	1	3 1013	NOT USED
N4	1	3 1015	NOT USED
N3	1	3 1017	NOT USED
N2	1	3 1021	2 7336
N1	1	3 1023	NOT USED
ISTOP	1	3 1123	NOT USED
N	1	3 1125	2 7344
NUMBER	1	3 1131	NOT USED
KOOK	1	3 1206	NOT USED

NNUMB	1	3 1213	NOT USED
NUMB	1	3 1215	NOT USED
I	1	3 1221	2 7352
IO	0	3 7775	2 7364

FLOATING POINT VARIABLES

IDENT	LEVEL	OBJECT CODE LOCATION
D3DW	4	2 7730
D2DW	4	2 7733
DDW	4	2 7745
XCK	3	2 7764
CORECT	3	2 7767
CAD	2	2 7774
SAD	2	3 0001
XMW	1	3 0221
PMAX	1	3 0253
XMAX	1	3 0265

R	1	3 0362			
RZ	1	3 0443			
SZ	1	3 0446			
S	1	3 0451			
ZEL	1	3 0465			
XT	1	3 0470			
XV	1	3 0473			
TAREA	1	3 0537			
RAREA	1	3 0542			
CCF	1	3 0772			
HCF	1	3 1000			
XP4	1	3 1054			
XP3	1	3 1062			
XP2	1	3 1070			
XP1	1	3 1076			
Q	1	3 1107			
EC	1	3 1137			
EH	1	3 1142			
BGC	1	3 1145			
BGH	1	3 1150			
CC	1	3 1153			
HC	1	3 1156			
B	1	3 1161			
A	1	3 1164			
XMARK	1	3 1210			

INTEGER ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
MAX	1	3 1677	1		
MAXC	1	3 1507	1		

FLOATING POINT ARRAYS

IDENT	LEVEL	OBJECT CODE LOCATION	DIMENSION	DIM1	DIM2
U	1	3 1223	1		
Z	1	3 1261	1		
DDW	1	3 5477	1		
XAXH	1	3 1317	1		
DC	1	3 1317	1		
GDH	1	3 2257	1		
AREAH	1	3 1507	1		
SDC	1	3 1507	1		
BAD	1	3 1677	2	4	
SDH	1	3 1677	1		
C2	1	3 2067	1		
DH	1	3 2067	1		
XW	1	3 2257	1		
QC	1	3 2257	1		
S20W	1	3 2447	1		
AI	1	3 2447	1		
GL	1	3 2447	1		
QH	1	3 2447	1		
X	1	3 2637	1		
ZC	1	3 2637	1		
W	1	3 3027	1		
ZH	1	3 3027	1		
H	1	3 3217	1		
DW	1	3 3217	1		
C1	1	3 5477	1		

C	1	3 5477	1
DX	1	3 5477	1

CONSTANTS

VALUE	OBJECT CODE LOCATION
0.27070000 E 01	2 7740
30	2 7750
0.12500000 E 01	2 7756
0.90000000 E 00	2 7761
0.11313280 E-03	3 0210
0.40000000 E 00	3 0213
0.52800000 E-01	3 0216
0.34600000 E 00	3 0224
0.88200000 E-01	3 0227
0.14100000 E 02	3 0262
20	3 0272
0.60000000 E 02	3 0274
0.49000000 E 02	3 0277
0.50000000 E 02	3 0302
50	3 0330
0.60000000 E 01	3 0332
6	3 0335
0.49000000 E 01	3 0337
0.20000000 E 02	3 0342
0.30000000 E 02	3 0365
0.15000000 E 01	3 0377
0.15000000 E 02	3 0402
0.75000000 E 01	3 0405
0.15000000 E 03	3 0426
0.10000000 E 00	3 0440
360	3 0454
0.25000000 E 01	3 0462
0.10000000 E 02	3 0526
0.10000000 E 03	3 0531
0.23030000 E 01	3 0534
0.10000000 E 01	3 0775
2	3 1025
0.0	3 1027
0.31000000 E-01	3 1032
0.25000000 E 00	3 1035
0.15000000 E 00	3 1040
0.75000000 E-01	3 1043
0.50000000 E 00	3 1046
0.30000000 E 00	3 1051
0.20000000 E 01	3 1057
0.12000000 E 01	3 1065
0.60000000 E 00	3 1073
0.50000000 E 01	3 1101
0.40000000 E 02	3 1104
0	3 1127
7	3 1173
3	3 1217
8	3 7757
5	3 7761
4	3 7763
400	3 7765
10	3 7767
40	3 7771
1	3 7773

SUBPROGRAMS

IDENT	LEVEL	OBJECT CODE LOCATION
MAXY	4	2 6366
CHECK	3	2 6126
SOLVE	2	2 5715

INTEGER VARIABLES USED AS SUBPROGRAM ARGUMENTS

IDENT	LEVEL	ERASABLE LOCATION	UP SUBROUTINE
N6	4	0 0004	NOT USED
N5	4	0 0003	NOT USED
N4	4	0 0002	NOT USED
L	3	0 0005	2 7134
J2	3	0 0003	2 7142
J	3	0 0002	2 7150
KCOL	2	0 0003	NOT USED
KROW	2	0 0002	NOT USED

INTEGER ARRAYS USED AS SUBPROGRAM ARGUMENTS

IDENT	LEVEL	ERASABLE LOCATION	DIMENSION	DIM1	DIM2
MAX	4	0 0005	1		

FLOATING POINT ARRAYS USED AS SUBPROGRAM ARGUMENTS

IDENT	LEVEL	ERASABLE LOCATION	DIMENSION	DIM1	DIM2
DW	4	0 0001	1		
C1	3	0 0004	1		
DH	3	0 0001	1		
BAD	2	0 0001	2	4	

STATEMENT NUMBERS

IDENT	LEVEL	OBJECT CODE LOCATION
214	4	2 6630
211	4	2 6576
208	4	2 6544
2080	4	2 6530
205	4	2 6514
202	4	2 6505
213	4	2 6647
203	4	2 6412
479	3	2 6317
478	3	2 6344
470	3	2 6365
474	3	2 6216
475	3	2 6243
476	3	2 6171
477	3	2 6267
21722	2	2 6032
21721	2	2 5762
21720	2	2 6075
600	1	2 5705
599	1	2 5676
67	1	2 5556
66	1	2 5544
21781	1	2 5520
21792	1	2 5464

21791	1	2 5444
2179	1	2 5434
2178	1	2 5454
2177	1	2 5326
2176	1	2 5306
2175	1	2 5276
2174	1	2 5316
77	1	2 4761
2173	1	2 5041
76	1	2 4700
75	1	2 4602
2171	1	2 4550
2170	1	2 5566
74	1	2 4404
73	1	2 4352
72	1	2 4426
21702	1	2 4310
21701	1	2 4222
21703	1	2 4346
217	1	2 4134
21705	1	2 4272
21704	1	2 4204
21706	1	2 4116
216	1	2 4051
601	1	2 5672
2166	1	2 4033
2152	1	2 4025
2151	1	2 4014
21	1	2 3762
215	1	2 4004
2010	1	2 3646
321	1	2 3610
322	1	2 3620
316	1	2 3531
319	1	2 3500
320	1	2 3537
204	1	2 3630
318	1	2 3423
3170	1	2 3413
317	1	2 3405
3150	1	2 3354
315	1	2 3364
3010	1	2 3226
301	1	2 3210
2007	1	2 3136
2006	1	2 3174
2005	1	2 3100
201	1	2 3074
50	1	2 2634
53	1	2 3070
2004	1	2 2310
20	1	2 2145
31	1	2 2504
30	1	2 2073
19	1	2 1773
1388	1	2 1505
426	1	2 1425
425	1	2 1415
424	1	2 1437
423	1	2 1447

1882	1	2 1340
1883	1	2 1330
1884	1	2 1351
422	1	2 1363
421	1	2 1276
189	1	2 1240
188	1	2 0730
573	1	2 0472
571	1	2 0326
48	1	2 0240
47	1	2 0224
570	1	2 0700
190	1	2 1615
18	1	1 7772
500	1	2 0041
118	1	1 7745
17	1	1 7661
179	1	1 7725
1378	1	1 7515
413	1	1 7435
412	1	1 7425
411	1	1 7447
420	1	1 7457
1785	1	1 7350
1279	1	1 7340
1280	1	1 7361
410	1	1 7373
409	1	1 7306
178	1	1 7252
177	1	1 7014
473	1	1 6556
471	1	1 6412
38	1	1 6324
37	1	1 6310
470	1	1 6764
400	1	1 6111
117	1	1 7635
28	1	1 6047
16	1	1 6017
29	1	1 6071
15	1	1 5751
26	1	1 5733
14	1	1 5720
11	1	1 5666
12	1	1 5650
13	1	1 6003
88	1	1 5413
7	1	1 5336
5	1	1 5255
3	1	1 5145
25	1	1 4751
24	1	1 4657
23	1	1 4715
1	1	1 4575
22	1	1 4574
92	1	1 4442
0	1	1 4433

FORMAT STATEMENTS

IDENT	LEVEL	OBJECT CODE LOCATION

221	1	3 0006
220	1	3 0035
219	1	3 0115
218	1	3 0141
2155	1	3 0232
52	1	3 0305
51	1	3 0321
2000	1	3 0345
2003	1	3 0353
2002	1	3 0370
1119	1	3 0410
119	1	3 0431
10	1	3 0545
9	1	3 0633
8	1	3 0752
2	1	3 1003
112	1	3 1112
111	1	3 1133
110	1	3 1167
91	1	3 1175

LIBRARY FUNCTIONS

IDENT	LEVEL	OBJECT CODE LOCATION
(16120		0 7264
(R405I		0 7340
(R162I		0 7424
PLOTTA		1 0001
INPUT		1 1725
A**I		1 2411
I**J		1 2501
ExPF		1 2553
LOGF		1 2773
SQRTF		1 3151
MDUMPF		1 3301
PLOTF		1 4047
XFILEF		1 4353

ERASABLE STORAGE 2 7375 TO 2 7730

VIII. LITERATURE CITED

VIII. LITERATURE CITED

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