The Protein Covalemently Linked to the 5' End
of Poliovirus RNA

by

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THE PROTEIN COVALENTLY LINKED TO THE 5' END
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ABSTRACT

Purification and partial characterization of the poliovirus RNA-linked protein (VPg) are described. VPg has been freed from the RNA by ribonuclease digestion and phenol extraction. Gel filtration chromatography of VPg-pUP (labelled with \(^{32}\text{P}\)) in 0.5% sodium dodecyl sulfate or 6 M guanidine HCl indicates that it has a molecular weight of about 12,000. VPg is bound to the 5' end of poliovirus RNA by a phosphodiester bond between a tyrosine residue in the VPg molecule and the 5'-terminal uridine. After acid hydrolysis of \([^{3}\text{H}]\)tyrosine-labelled VPg-pU, free tyrosine can be released by venom phosphodiesterase. Acid hydrolysis of VPg-p labelled with either \(^{32}\text{P}\) or \([^{3}\text{H}]\)tyrosine yields tyrosine-phosphate. There appears to be only 1 tyrosine residue per VPg molecule. VPg can be recovered from poliovirus RNA chains varying in length from 7,500 nucleotides (full-sized RNA) to about 500 nucleotides. No other type of 5' terminus can be demonstrated on nascent RNA, and the yield of VPg is consistent with one molecule of the protein on each nascent chain. These results are consistent with the concept that the protein is added to the 5' end of the growing RNA chains at a very early stage, possibly as a primer of RNA synthesis.
The 5' terminal protein (VPg) on poliovirion RNA can be removed by cell-free extracts from a variety of uninfected cells. This soluble enzymatic activity is found in both nuclear and cytoplasmic extracts of HeLa cells and is activated by Mg$^{++}$. The enzyme activity cleaves the tyrosine-phosphate bond that links the protein to the RNA. In a partially purified form it has insufficient nonspecific protease or nuclease activity to account for its action. The existence of this enzyme implies that removal of VPg from poliovirus RNA by a cellular enzyme (unlinking enzyme) is a normal event during polio infection. Specific hypotheses for the roles of VPg and unlinking enzyme in infected cells are discussed. The substrate specificity of this enzyme has been investigated in vitro. VPg-pU or VPg-pUp is resistant to cleavage under conditions where either VPg or the protease-K-resistant RNA-bound oligopeptide fragment of VPg is removed from full length poliovirus RNA. Possible roles for unlinking enzyme in uninfected cells are discussed.

Thesis Advisor: David Baltimore, American Cancer Society Professor of Microbiology.
CHAPTER I: INTRODUCTION AND BACKGROUND

1) GENERAL INTRODUCTION TO POLIOVIRUS

A. Poliomyelitis

Poliomyelitis is a disease caused by poliovirus in humans and Rhesus monkeys. In 1908, Karl Landsteiner was the first to isolate the infectious agent for poliomyelitis from infected human spinal cord. Landsteiner was able to induce spinal cord lesions in monkeys with the isolated virus. Since that time, it has been shown that 3-4% of poliomyelitis cases result in paralysis, while the rest of infections are restricted to the enteric tract.

Poliomyelitis can be grown in tissue culture, enabling large quantities to be produced for vaccination of humans. The Salk Vaccine, using inactivated virus, and the Sabin Vaccine, using live, attenuated virus, have been successfully used in the prevention of poliomyelitis among modern populations. While the method of prevention of the disease is well established, much study has been devoted to the molecular aspects of viral replication within infected tissue in hope of understanding the virus and the disease process. Fundamental and detailed understanding of poliovirus replication may allow the development of methods for the inhibition of infection by related viruses which are not as easily controlled by vaccination.

B. The Picornaviruses

Poliomyelitis is a member of the Picornaviridae family of animal viruses. The following is a summary of the thorough discussion of the definition and classification of picornaviruses by Cooper, et al., (1978). Criteria for the classification of a virus as belonging to the picornavirus family are morphology, physiochemical properties, and strategy of replication. All picornaviruses are small (less than 30nm diameter), spherical,
noneveloped virus particles containing RNA and protein. Picornavirus RNA is infectious. The virions are typically 24-28 nm diameter, sediment at 150-156S, and band in CsCl at 1.33-1.45 gm/cm$^3$. A large number of viruses (Cooper, et al., 1978) with hosts included among invertebrates as well as vertebrates, technically qualify as picornaviruses by the above criteria. These viruses have been further classified into four subfamilial genera: the enteroviruses, cardioviruses, rhinoviruses, and aphthoviruses. Genera are distinguished by sensitivity to acid, density in CsCl, and type of disease caused in the host. It is a general property of picornaviruses that most infections are not lethal to the host. Survival of the host population is, of course, necessary for the survival of the virus.

Poliovirus is a member of the genus of enteroviruses, so named because they infect the enteric tract of their hosts. Paralysis occasionally results from enterovirus infection of nervous tissue. The many species of enteroviruses include murine poliovirus, Bee acute paralysis virus, and others.

The cardioviruses include encephalomyocarditis virus (EMC) and mengovirus. Cardioviruses infect many tissues and are often pathological or lethal in mice or hamsters.

Rhinoviruses, which include human rhinovirus and bovine rhinovirus, infect ciliated epithelium of upper and lower respiratory tract, causing colds.

Aphthoviruses naturally infect cloven-footed animals, but will infect most laboratory animals. Infection is often fatal in young animals due to fever and muscle lesions.

In general, the picornaviruses are structurally and biochemically nearly identical. The following discussion refers chiefly to poliovirus, but what is described for poliovirus can be considered true for other picornaviruses.
C. Poliovirus Composition and Structure

The polio virion is a ribonucleoprotein particle of spherical symmetry with 60 identical protein subunits arranged in icosahedral geometry (Dunker, 1974). Purified virions have been shown to contain no phospholipid (Shaffrad, Shwerdt, 1959), and no D-galactose or N-acetyl-D-glucosamine (Drzeniek, Bilello, 1974). The protein component of mature virions includes four major polypeptides; VP1 \((\text{mw} = 35,000)\), VP2 \((\text{mw} = 28,000)\), VP3 \((\text{mw} = 24,000)\), and VP4 \((\text{mw} = 8,000)\). There are approximately 60 copies each of VP1, VP2, VP3, and VP4 per virion, so each of the 60 protein subunits of the virion probably contains one copy of each of the four viral proteins.

In addition to the major structural proteins, there are minor protein components of the virion. VP0, the precursor to VP2 and VP4 is present in 1-2 copies per virion (Maizel, et al., 1967). There are also several small viral proteins (SVP) associated with purified virions. \(^{32}\text{P}\) labelled virions contain a protein labelled with radioactive phosphate (VPp) which comigrates on SDS gels with the noncapsid viral protein NCVPX (Naomi Guttman, MIT PhD thesis). It is not known whether VPp is a phosphorylated form of NCVPX contained in the virion, or a phosphorylated form of one of the capsid proteins with altered mobility due to its phosphate content.

D. The Poliovirus RNA Genome

The RNA molecule contained within the polio virion is \(2.6 \times 10^6\) daltons molecular weight, or \(7500 \pm 400\) nucleotides (Granboulan and Girard, 1969). The RNA sediments at 35S on sucrose gradients (Tannock, et al., 1974), and contains 3' terminal poly(A) (Yogo and Wimmer, 1972). Virion RNA is infectious (Colter, et al., 1957) but removal of poly(A) diminishes infectivity (Spector and Baltimore, 1974), implying a role for
poly(A) in polio replication. Virion RNA can act as messenger RNA to direct the synthesis of polio specific proteins. Poliovirus is therefore a positive stranded virus, referring to the coding sense of the virion RNA.

Virion RNA contains a protein (VPg) covalently attached to its 5' end (Flanegan, et al., 1977). This protein and its function will be discussed in detail in subsequent sections and chapters. Intracellular forms of poliovirus RNA which are not found in virions will be discussed in sections devoted to the viral processes with which they are associated.

2) THE POLIOVIRUS REPLICATION CYCLE

A. Introduction

Poliovirus is lytic virus. Its one step growth cycle is characterized by rapid replication within the host cytoplasm followed after 6-12 hours of infection by lysis of the cell and release of progeny virus. In an infected animal, the pathology of poliomyelitis is caused either directly or indirectly by this rapid destruction of infected tissue and spread of infectious particles.

The molecular and biochemical details of the poliovirus life cycle have been extensively studied using infected tissue culture cells, such as HeLa cells. There are at least two reasons for these studies. First, the ability to synchronously infect mass cultures of cells with poliovirus allows the biochemical study of the synthesis and assembly of viral macromolecules in the host cytoplasm. The fact that the various picornaviruses are essentially identical means that the results of studies of poliovirus also apply toward the understanding of all picornaviruses. Second, poliovirus, upon infecting a host cell, specifically perturbs various cellular functions. These perturbations offer the opportunity to identify and study the elements of certain cellular processes. For
example, poliovirus is able to shut off host cell protein synthesis. By understanding the mechanisms of shut-off, it will be possible to identify crucial elements of cellular protein synthesis. Furthermore, a general property of infection by poliovirus and other picornaviruses is that certain host functions are recruited for viral purposes. This recruitment may involve specific modification of a host structure, or its physical association or cooperation with a viral-coded function. Some examples of such host-viral interactions in polio infected cells will be described below. Studies of these phenomena afford the opportunity to, not only further understand the strategy of viral infection, but also to identify important cellular structural and functional elements.

The following sections contain a brief summary of the known events and processes that occur during poliovirus infection of cultured cells and some of what is known or not known about the viral and cellular participants. The order in which topics are discussed is not meant to imply a strict temporal order of biochemical events after infection. Temporal relationships will be mentioned where relevant.

B. Poliovirus Binding and Alteration by Host Cells

Poliovirus will infect human and monkey tissues, but not dog, cat, rabbit or mouse. All these tissues, however, are susceptible to infection by polio RNA. Therefore, host restriction is on the level of entry of the virus into the cell (Holland, McLaren, Syverton, 1959).

Poliovirions specifically bind to receptors on the surface of Hela cells. This is the initial event of infection of a cell. This binding occurs reversibly at 22°C or below, without alteration in properties of the bound virus particles. Reversibly bound virions can be recovered from the cell without loss of infectivity by treatments such as 6M LiCl
(Holland, 1962), detergents (Fenwick and Cooper, 1962) or proteases (Zajac and Crowell, 1965). The release of infectious virus from the cell by high salt or protease treatment implies that virion binding is to a protein containing receptor on the cell surface. The gene coding for the poliovirus receptor has been mapped to human chromosome 19 by virion binding studies of mouse-human hybrid cell lines (Wang, et al., 1970).

Tissues such as intestine, spinal cord and brain, which are sensitive to poliovirus infection in a susceptible animal, also show specific binding in culture. Tissues which are normally resistant to infection, including heart, lung, skin and kidney show no specific binding (Holland, 1961; Holland and McLaren, 1961). Cells from tissues normally resistant to poliovirus infection have been reported to develop poliovirus receptors upon culture (Holland, 1961).

Poliovirus incubated with sensitive cells at 37°C undergoes irreversible alteration. This alteration is characterized by the inability to recover infectious particles by chemical means. This alteration is accompanied by elution of noninfectious viral material from the cell. Most of viral material bound to cells at 4°C is eluted at 37°C. This fact may explain the high ratio of poliovirus particles to plague-forming units (Darnell and Eagle, 1960).

The irreversibly altered poliovirus is found in several forms. There are several assays for virion alteration by cells, and each assay measures a particular form of altered particle. Irreversible loss of infectivity reflects total breakdown of virus. Sensitivity of viral RNA to a mixture of protease and ribonuclease (Chan and Black, 1970), or to ribonuclease alone, measures amount of partially uncoated particle, or amount of completely uncoated viral RNA, respectively. The generation
of an altered particle defined by both sensitivity to SDS and ribonuclease, and shifted sedimentation coefficient correlates with the disappearance of infectivity (Guttman, 1976). This altered particle sediments at 130S on sucrose gradients. The poliovirus receptor molecule and alteration activity was found associated with the plasma membrane of the cell since virus bound to isolated plasma membrane is altered to the 130S particle at 37°. The 130S particle lacks VP4 and is considered an intermediate in the complete uncoating and internalization of poliovirus RNA. The normal functions of the poliovirus receptor and alteration activity in uninfected cells have not been determined.

C. Virus-Host Interactions

The first interaction between a host cell and an infecting poliovirion is the binding and uncoating process described above. Upon initiating infection, poliovirus causes several detectable changes in the host cell. These changes serve to facilitate the full utilization of cellular machinery for viral purposes.

Nuclear RNA synthesis is suppressed soon after polio infection, and initiation of new DNA synthesis (Salzman, et al., 1959) and entry into mitosis (Hand and Tamm, 1972) are inhibited. Poliovirus replication proceeds normally in cells treated with Actinomycin-D (Reich, et al., 1961), or in enucleated cells (Pollack and Goldman, 1973), so cellular RNA or DNA synthesis is not necessary for poliovirus replication.

Synthesis of smooth membranes is stimulated by poliovirus infection (Mosser, et al., 1972). Poliovirus RNA synthesis occurs in association with smooth membranes, so increase in quantity of these structures would be advantageous for RNA replication.

Cellular protein synthesis is rapidly shut off after poliovirus
infection. Viral RNA replication is not necessary for this shut-off (Penman and Summers, 1963) so the active agent may act catalytically. No detectable alteration in the size, poly(A) content, or 5' ends structure of cellular mRNA's occurs (Willems and Penman, 1966; Fernandez-Munoz and Darnell, 1976). Inhibition is thought to be at the level of initiation (Leibowitz and Penman, 1971). One model to explain how polio RNA is translated in infected cells, but cellular mRNA is not, is that poliovirus codes for a protein which specifically alters the translation machinery of the cell. This alteration creates a block in some step essential for the initiation of cellular mRNA's. Polio circumvents that step through some structural difference between polio mRNA and cellular mRNA. There is some evidence consistent with this model. Cell mRNA's have 7-methyl-Guanosine containing caps at their 5' termini. Polio mRNA contains no cap structure, but terminates with pUp (Hewlett, et al., 1976). Furthermore, extracts of polio infected Hela cells contain an agent capable of inhibiting the in vitro translation of capped VSV mRNA, but which does not affect polio translation (Rose, et al., 1978). The inhibitory effect of this activity was overcome by the addition of a preparation of eukaryotic initiation factor eIF-4B. It is not known for certain which component of the eIF-4B preparation was responsible for the recovery of capped VSV mRNA translation. It is clear that extracts of poliovirus infected cells contain an activity which inactivates some component of normal cells which is necessary for cellular mRNA translation, but not for polio translation. The inhibitor activity is not found in uninfected extracts and is probably virus-coded. Further evidence that the shut-off factor is poliovirus comes from the report by Cooper (1973) that some poliovirus mutants, temperature sensitive for virion structural
proteins, were also temperature sensitive for host protein synthesis shut-off. However, these mutants were very leaky, and failed to suppress host protein synthesis at high temperature only when viral RNA synthesis was inhibited by guanidine.

D. Viral RNA Synthesis

Polioviral RNA can be radioactively labelled during infection under conditions where host RNA synthesis is suppressed. For the first three hours of infection, viral RNA accumulates exponentially, and then synthesis continues linearly for about one hour (Baltimore, Girard and Darnell, 1966). Newly made RNA is found in a complex called the replicative intermediate (RI). The nascent polio RNA associated with RI was found to be mostly of positive polarity (Baltimore, 1968). Phenol extracted RI is partially double stranded, and partially single stranded, and contains poly(A) and poly(U) (Yogo and Wimmer, 1975). The RI is found associated with smooth membranes (Caliguiri and Tamm, 1970), and sediments at 75 to 265S on sucrose gradients after release from membranes (Caliguiri and Mosser, 1971).

The form of RI released from membranes by detergent was found to contain the viral proteins NCVPX, NCVP4, and its precursor, NCVP2 (Butterworth, et al., 1976). Another group reported that NCVP4 was the only major viral polypeptide (Lundquist, et al., 1974). Flanegan and Baltimore (1978) found one major viral protein, p63, associated with solublized replicating RNA after ribonuclease treatment. p63 is probably the same polypeptide as the one called NCVP4 by others. NCVPX was proposed to play a role in attachment of the replication complex to membranes (Butterworth, et al., 1976). p63 (NCVP4) copurifies with poliovirus poly(U) polymerase activity from infected cells (Flanegan and Baltimore, 1979). The fact that p63 was found firmly attached to replication complex
indicated that p63 may be active in poliovirus RNA synthesis. p63 and its precursor, NCVP2, both copurify from the soluble fraction of infected cells with the activity able to replicate polio RNA in vitro (DasGupta personal communication). Therefore, NCVP2 may also play a role in polio RNA synthesis. The mechanism of initiation of polio RNA synthesis in vivo is not known. Purified polio replicase seems to initiate de novo and at random in vitro (M. Baron, personal communication), and shows only slight preference for polio RNA as template (DasGupta, et al., 1979). The replicase can synthesize nearly full length copies of polio RNA when oligo(U) is included as a primer (Flanegan, personal communication). Specific initiation in vivo may use an analogous primer. The possible role of the 5' terminal protein (VPg) of poliovirus in such a priming event will also be discussed in later chapters.

At least one host factor is necessary for in vitro activity of polio replicase (DasGupta and Baltimore, personal communication). This factor has been partially purified but its function in normal cells is not known, nor is its precise role in polio RNA synthesis understood.

Newly synthesized viral RNA can serve as template for RNA synthesis, act as mRNA, or be encapsidated. It is not known what factors determine the fate of a new viral RNA molecule. One factor may be 5' terminal VPg, which is present on virion RNA, but not on polysomal RNA. This protein and its possible functions will be discussed in subsequent sections and chapters.

E. Synthesis and Processing of Poliovirus Proteins

Poliovirus 35S RNA has coding capacity for approximately 270,000 daltons of protein. The viral polypeptides labelled under conditions where the host protein synthesis is suppressed range in molecular weight
from 6,000 to 100,000 (Summers, et al., 1965). The sum of their molecular weights exceeds 270,000 daltons. Pulse-chase experiments demonstrated that larger proteins could be chased into smaller proteins (Summers and Maizel, 1968). Inhibition of proteolytic cleavage allowed the buildup of larger polypeptides, the largest of molecular weight 210,000 daltons (Jacobson and Baltimore, 1968). Polio RNA is therefore polycistronic, with all the various polypeptides being derived by proteolytic cleavage of one large precursor (Jacobson, et al., 1970).

Pactamycin mapping experiments determined that the order of the three major cleavage products was NCVP1a, NCVPX, NCVP2 (Taber, et al., 1971). Tryptic peptide mapping, however, failed to show that NCVPX shares peptides with the 210,000 dalton precursor(p210), while NCVP1a and NCVP2 do share peptides with p210 (Abraham and Cooper, 1975). This presents an apparent paradox, since NCVPX mapped between NCVP1a and NCVP2. The order of polypeptides determined by pactamycin mapping is based on the assumption of one initiation site. Therefore one explanation for the NCVPX paradox is that NCVPX is translated from a separate initiation site than p210. On the other hand, Reuckert, et al., (1979) report that the human rhinovirus 1A protein p38, which is homologous to poliovirus NCVPX, shares tryptic peptides with a 146,000 dalton precursor polypeptide. This p146 also contains sequences of p92, which is homologous to the poliovirus virion protein precursor NCVP1a. Poliovirus NCVPX may also be derived from a larger precursor, but comparison of tryptic peptide patterns may have been difficult due to some modification of NCVPX (such as phosphorylation).

There is conflicting evidence for the number of initiation sites for poliovirus protein synthesis. Exogenously added poliovirus RNA directed the incorporation of f-met into one polypeptide in a Hela cell-free
system. That labelled polypeptide was identical to NCVP1a. Only one f-met labelled tryptic peptide was resolved by pH 3.5 paper electrophoresis (Villa-Komaroff, et al., 1975). Ehrenfeld (1977) reported that extracts of polio infected cells are able to initiate translation of endogenous RNA at two sites, producing two distinct f-met labelled peptides. The f-met labelled proteins are identical in size to NCVP1a and VP4. The relative degree of initiation at each of these sites is sensitive to Mg⁺⁺ concentration. These conflicting observations may be explained by differences in the translation systems used. It is not known which result more accurately reflects in vivo processes. The smaller f-met labelled polypeptide observed by Ehrenfeld is approximately the size of VPg. It is possible that VPg is translated from a separate initiation site than the majority of polioviral proteins.

F. Virion Assembly

The viral proteins VP0, VP1, and VP3, as well as their precursor polypeptide, NCVP1a, are found in 14S complexes in infected cells. Radioactive amino acid first labels the 14S structure, then the 74S procapsid, which contain 60 copies each of VP0, VP1, and VP3 (Maizel, et al., 196`). Procapsids, which contain no RNA, accumulate in the presence of the viral RNA synthesis inhibitor guanidine. Removal of guanidine allows procapsids to be chased into virions (Jacobson and Baltimore, 1968).

Ribonuclease resistant polio RNA can be found in a structure containing only VP0, VP1 and VP3. This structure is called the provirion (Fernandez-Tomas and Baltimore, 1972). Provirion can be generated in extracts of infected cells (Fernandez-Tomas, et al., 1973). In Vitro labelled polio RNA was found to be associated with material sedimenting
like provirion (Yin, 1977), and procapsid was found on membrane-bound replication complex. This procapsid was released by ribonuclease treatment. Therefore, it was suggested that procapsid binds to nascent poliovirus RNA molecules to initiate encapsidation. The intermediate product of encapsidation would be provirion, which is thought to undergo proteolytic cleavage of VP0 to VP2 and VP4 to form mature virions.

The structural basis for the specific association of procapsid with polio plus strands is not known. One unique feature of virion polio RNA which distinguishes it from host cell RNA as well as from polio mRNA is the presence of a 5' terminal protein which is discussed in the next two sections.

3) **5' TERMINAL PROTEIN-NUCLEIC ACID COVALENT COMPLEXES**

**A. Viral Systems**

The virion RNA's of several picornaviruses, including poliovirus (Flanagan, et al., 1977; Nomoto, et al., 1976), EMC (Golini, et al., 1978) and FMDV (Sanger, et al., 1977) have a protein (VPg) covalently linked to their 5' terminus. This protein appears to be virus-specified (Nomoto, et al., 1977). Another RNA virus found to contain terminal protein is the plus-stranded plant virus Cowpea mosaic virus (CPMV) which has a 5-10 K molecular weight protein covalently bound to pUpA. . . at the 5' end to virion RNA molecules (Stanley, et al., 1978). The infectivity of the purified RNA genome of nepovirus (Harrison and Barker, 1978) and calcivirus (Burroughs, 1978) is sensitive to protease treatment. It was therefore suggested for each of these viruses that a protein, necessary for initiation of infection, is tightly bound to the RNA. The 5' ends of these RNA's lack any capped ends or other identifiable 5' terminal structures, so the protein may be 5' terminal. The calcivirus
RNA-bound protein was labelled with lysine and its molecular weight measured approximately 10,000 daltons.

Adenovirus virion DNA (Rekosh, et al., 1977) and Adenovirus newly made DNA (Coombs, et al., 1978) have 5' terminal covalently bound protein. Evidence for this terminal protein includes the following. The 5' ends of Adenovirus DNA are resistant to $\lambda$ 5'- exonuclease, and cannot be phosphorylated by T-4 polynucleotide kinase after treatment with phosphatase (Carusi, 1977). Protease sensitive circular structures can be visualized by electron microscopy (Rekosh, et al., 1977). The terminal restriction fragments of adenovirus do not enter agarose gels during electrophoresis unless treated with protease. Furthermore, in vivo pulse-labelled adenovirus DNA molecules, intracellular parental molecules, and virion DNA, bind to glass fiber filters via the terminal protein (Coombs, et al., 1978). A role for terminal protein as a primer for Adenovirus DNA synthesis has been suggested (Rekosh et al., 1977). Minute virus of Mouse (MMV), a parvovirus, seems to also have protein covalently bound to at least a subpopulation of intracellular viral DNA (Marie Chow, personal communication). The protein is bound to the terminal restriction fragments of molecules active in DNA synthesis. MMV virions contain a single-stranded DNA genome which seems to lack the terminal protein. Therefore, the possibility exists that the MMV terminal protein is enzymatically removed prior to encapsidation. This terminal protein has not been shown to be viral coded, and may be of cellular origin (Marie Chow, personal communication).

A covalent protein-DNA complex has also been reported in SV40 (Griffith, et al., 1975). The protein seems to be located at the origin of replication, as judged by electron microscopy.
Strong evidence for the role of 5' terminal covalently bound protein in viral DNA replication is provided in the case of φ29, a *Bacillus subtilis* phage. The double stranded φ29 virion DNA molecule is held in a circular conformation by a protein (p3) covalently bound to the 5' end of each strand (Ito, Kawamura, Yanofsky, 1976). The gene for this protein has been identified as cistron 3 of the virus. This gene is expressed early in infection, its expression is required continuously for DNA synthesis, and terminal protein is required for infectivity of the virion DNA (Salas, et al., 1978). These investigators suggest that p3 plays a central function in DNA synthesis, perhaps acting as a primer, and also propose a role for p3 in packaging of φ29 DNA.

The φX174 cistron A protein was shown to be bound to the 5'-phosphoryl end of a single-stranded break in viral DNA (Langeveld, et al., 1978; Ikeda, et al., 1976). Electron microscopic visualization of protein-DNA complexes showed that the cistron A protein is bound to the origin of replication of replicative form II DNA. Several proposals for a role of this protein in φX174 DNA replication were made (Eisenberg, et al., 1977).

The functions of protein covalently bound to viral nucleic acid may vary from system to system. A preliminary distinction can be made between those terminal proteins which are required for infectivity of the nucleic acid, such as adenovirus, φ29, nepovirus, and calcivirus terminal proteins, and those terminal proteins not required for infectivity, such as the terminal protein of CPMV, MMV terminal protein, and picornavirus VPg (Nomoto, et al., 1977; this thesis). Those terminal proteins which are required for infectivity may have to remain attached to the nucleic acid in order to interact in some way with cellular or early viral coded functions and thereby participate in the initiation of the replicative
cycle. Those terminal proteins which are not required for infectivity may normally be removed from infecting nucleic acid. Terminal proteins of this class may well participate in nucleic acid synthesis, or perhaps be involved in the encapsidation of progeny genomes, but need not remain bound to the parental nucleic acid in order to function.

B. Non-Viral Systems

The ciliogenic facors Col E1 and Col E2 contain a protein covalently bound to the 5' end of one of the two DNA strands at the origin of replication (Blair and Helinsky, 1975; Lovett and Helinsky, 1975; Guiney and Helinsky, 1975).

Thus far, no non-viral RNA molecules of prokaryotes or eukaryotes have been shown to contain 5' terminal protein. There is a report of evidence for protein tightly associated with Hela chromosomal DNA (Coombs, et al, 1978), but these structures have not been characterized. Several prokaryotic proteins are able to cleave specific phosphodiester bonds and in the process become covalently bound to the 5' phosphoryl end of the break. These include plasmid relaxation complex (Clewell and Helinsky, 1969) and E. Coli DNA gyrase (Sugino, et al, 1978; Gellert, et al, 1978).

C. Chemical Nature of Protein-Nucleic acid Linkages

T₄ RNA ligase (Cranston, et al, 1974) and DNA ligase (Weiss, et al, 1968) form covalent ligase-AMP complexes in the presence of ATP. The AMP is linked to the enzyme molecule by a 5' phosphoramid linkage to a lysine side chain (Gumport and Lehman, 1971). E Coli glutamine synthetase and its regulatory protein PII are adenylylated and uridylylated, respectively, via a 5' phosphodiester linkage to tyrosine (Adler, et al, 1975). The omega protein of E Coli binds to the 5' phosphoryl end of a nick in duplex DNA via a
phosphodiester bond to tyrosine (Wang, personal communication). The genome RNA molecule of EMC virus, a picornavirus, has recently been found to be linked to its terminal protein by a tyrosine-phosphate linkage (Bogdanski, personal communication). Thus far, only in the above cases, and in the case of poliovirus, (this thesis; Ambros and Baltimore, 1978), is the precise nature of the protein–nucleic acid linkage known. Both the o29 (Salas, et al, 1978) and adenovirus (Carusi, 1977) linkages are sensitive to alkali, suggesting a phosphodiester bond.
The protein bound to Col E1 and Col E2 (Guiney and Helsinki, 1975), SV40 (Kasamatsu and Wu, 1976a) and 329 (Salas, et al., 1978) is stable to hydroxylamine treatment, a reagent which cleaves phosphoramid linkages (Shabarova, 1970).

4) THE ROLE OF VPg IN POLIOVIRUS INFECTION

The poliovirus 5' terminal protein (VPg) is linked to an oligonucleotide at the end of virion RNA with the sequence: VPg-pU-U-A-A-A-A-C-A-Gp . . . (Flanegan, et al., 1977; Nomoto, et al., 1977). The messenger RNA isolated from the polyribosomes of infected cells, however, lacks the protein and terminates at its 5' end in pU-U-A-A-A-A-C-A-Gp (Pettersson, et al., 1977). Thus, the only apparent difference between virion RNA and mRNA is the presence or absence of the protein. The protein is also found in preparation of polio double-stranded RNA (Nomoto, et al., 1977; Pettersson, et al., 1978), and replicative intermediate (RI) RNA (Flanegan, et al., 1977; Nomoto, et al., 1977), the structure in which new viral RNA is synthesized. This finding led to the suggestion that the protein, possibly linked to one or several nucleotides, might serve as a primer for initiating poliovirus RNA synthesis (Flanegan, et al., 1977; Nomoto, et al., 1977). Subsequently, the protein would be cleaved from the strands destined to become mRNA. RNA retaining the protein would be encapsidated into mature virions (Pettersson, et al., 1977).

The VPg of EMC RNA and the VPg of poliovirus grown in the same cell type are of different molecular weights. EMC virus grown in cells of different species contained identical VPg (Golini, et al., 1978). These experiments indicated that the size of 5' terminal protein of picornavirus is virus-specific, not cell-specific, and VPg is therefore not a cellular
protein. The fact that VPg can be labelled with radioactive amino acid at three hours after infection when all known host protein synthesis is suppressed also argues that VPg is virus-coded (Lee, et al., 1977).

The work described in subsequent chapters of this thesis comprises a characterization of some properties of poliovirus VPg, and the nature of the polio protein-nucleic acid linkage. Experiments are describes which pertain to the possible role(s) of VPg in polio infection. A Hela cell enzyme capable of processing newly made polio RNA into messenger RNA is described and partially characterized.
CHAPTER II:

A PROTEIN IS LINKED TO THE 5' END OF POLIOVIRUS RNA BY A PHOSPHODIESTER LINKAGE TO TYROSINE

1) INTRODUCTION

The single-stranded RNA genome of poliovirus is covalently bound through its 5'-terminal phosphate to a virus-specified protein (VPg) (Flanagan, et al., 1977; Nomoto, et al., 1976). To better understand the function of VPg in poliovirus replication, and the mechanism of formation and breakage of the protein-nucleic acid linkage, it is necessary to know the chemical nature of that linkage. The experiments in this chapter demonstrate that VPg is linked to the 5' terminus of poliovirus RNA through a phosphodiester bond to tyrosine. This chapter also contains the description of a method for rapid purification of VPg from virion RNA and infected cell RNA, and a molecular weight estimation for VPg.

2) METHODS AND MATERIALS AND EXPERIMENTAL PROCEDURE

A. Cells and Virus

HeLa cells were infected with type I poliovirus and virions were purified as previously described (Baltimore, et al., 1966). Virion RNA was labelled with \( ^{32}P \) and purified as described elsewhere (Hewlett, et al., 1976). Poliovirus was labelled with \( ^{3}H \) tyrosine by infecting \( 4 \times 10^8 \) HeLa cells in 100 ml of Earle's saline, supplemented with all amino acids except tyrosine and including 5% dialyzed fetal calf serum. Actinomycin D (5 \( \mu \)g/ml) was added 15 min postinfection and 5 mCi of \( ^{3}H \) tyrosine (40 to 50 Ci/m mole, New England Nuclear Corp.) was added 1 h postinfection. Virus was harvested at 6 h after infection and purified by high speed centrifugation and sodium dodecyl sulfate-sucrose gradient sedimentation.
Virions were lysed by extraction with phenol:chloroform:isoamyl alcohol (50:48:2) and 35S RNA was isolated by sedimentation through a 15 to 30% sucrose gradient in 0.5% sodium dodecyl sulfate, 0.1 M NaCl, 50 mM Tris, pH 7.5, 1 mM EDTA. Fractions were collected and a portion of each fraction was counted in Bray’s solution. RNA was recovered from the peak of [3H]tyrosine radioactivity by ethanol precipitation.

B. Enzymatic Digestion

RNA was digested with P1 nuclease (Rose and Lodish, 1976), a mixture of nucleases T1, T2, and pancreatic ribonuclease, or T1 nuclease alone (Flanegan, et al., 1977). The conditions for micrococcal nuclease digestion were 20 μg of carrier yeast tRNA, 150 units of nuclease (P-L Biochemicals) in 10 μl of 20 mM glycerine, pH 9.0, 5 mM CaCl₂, 37°C, 2 h. Proteinase K digestion was in 10 μl of 20 mM Tris, pH 7.5, 2 mM EDTA, 200 μg/ml of protease K, at 37°C for 2 h. The conditions for venom phosphodiesterase and bacterial alkaline phosphatase digestion are described elsewhere (Fish, et al., 1969).

C. Purification of VPg-pUp, VPg-pU, and VPg-p

Poliovirion RNA labelled with either [3H]tyrosine or 32P was digested with the appropriate nuclease. After digestion, the sample volume was adjusted to 50 μl with 20 mM Tris, pH 7.5, 2 mM EDTA and the digest was extracted twice with 50 μl of phenol:chloroform:isoamyl alcohol (50:48:2). The pooled organic phases plus interfaces were then washed four times with 100 μl of 20 mM Tris, 2 mM EDTA to remove contaminating ribonucleotides. The material contained in the organic phase and interface was precipitated with 10 volumes of acetone at -20°C. The precipitate was recovered by centrifugation, and the pellet was dried and dissolved in 50 mM Tris,
0.1 M NaCl, 1 mM EDTA buffer plus 0.5% sodium dodecyl sulfate. The material recovered from the phenol was chromatographed through a column of Sephadex G-25 (0.5 x 10 cm) and the void volume fractions were precipitated with acetone. This excluded material consisted of radioactive VPg attached to a phosphate or uridylate residue. Digestion of RNA with nucleases T1, T2, and A yields VPg-pUp (Flanegan, et al., 1977); P1 digestion yields VPg-pU (Flanegan, et al., 1977). Micrococcal nuclease digestion of VPg-pUp yields VPg-p.

D. Molecular Weight Estimation of VPg-pUp

Gel filtration of $^{32}$P-labelled VPg-pUp was carried out on a column (1.0 x 30 cm) of BioGel A-1.5m equilibrated with 0.5% sodium dodecyl sulfate, 50 mM Tris, 0.1 M NaCl, 1 mM EDTA. Blue dextran 2000, bromphenol blue, and 50 μg of cytochrome c were included as visible markers. For molecular weight determination, the column was calibrated with a sample containing $^{125}$I-labelled Moloney leukemia virus p12 and p30, 300 μg of insulin, 10 μg of cytochrome c, plus bromphenol blue and blue dextran 2000. $^{32}$P was detected by Cerenkov radiation, $^{125}$I by γ emission, cytochrome c by A$_{300}$ nm, bromphenol blue and blue dextran by A$_{600}$, and insulin by A$_{280}$. VPg was recovered from the peak fractions by acetone precipitation. Gel filtration chromatography of VPg-pUp in 6 M guanidine HCl, 0.1 M β-mercaptoethanol on a BioGel A-5m was exactly according to Fish et al. (1969) except that the column dimensions were 1.0 x 30 cm.

E. Acid Hydrolysis of VPg

VPg was hydrolyzed in sealed glass ampules under nitrogen in the presence of 20 μg of bovine serum albumin carrier protein in 200 μl of 2 M HCl at 110°C for 24 h (Evewleigh and Winter, 1970). The hydrolysate was freeze-dried and dissolved in water, and a portion was treated with
venom phosphodiesterase or alkaline phosphatase.

F. 3MM Paper Ionophoresis

Acid hydrolysates were spotted on a 34-cm length of Whatman No. 3MM paper and subjected to electrophoresis in pH 3.5 pyridine/acetic acid buffer (Barrell, 1971) at 2400 V for 1 h. Each lane was then cut into 0.5-cm slices, and each slice was soaked in 0.5 ml of water for 30 min and then counted in 10 ml of Bray's solution. 32P labelled samples were electrophoresed on 3MM paper in an identical manner and radioactivity detected by autoradiography using Kodak XR-5 film.

Electrophoretic markers of phosphoserine, phosphothreonine, and tyrosine (Sigma) were detected by spraying the dried Whatman No. 3MM paper with a 0.1% solution of ninhydrin in ethanol followed by heating at 65°C for 5 min.

G. Thin Layer Chromatography

[^3H]Tyrosine-labelled VPg-pU was prepared as described above. After the pU was removed by digestion with venom phosphodiesterase, the protein was hydrolyzed as described above, except the bovine serum albumin carrier was omitted. The hydolysate was freeze-dried and redissolved in 20μl of H2O plus 5μg of tyrosine carrier. The sample was spotted onto a cellulose-coated glass thin layer chromatography plate (10 x 10 cm) and two-dimensional development was carried out. First dimension, butanol:acetic acid:H2O, 18:2:5. Second dimension, butanol:pyridine:acetic acid:H2O, 32.5:29:5:20 (v/v) (Gibson, 1974). 2,5-Diphenyloxazole (PPO) was included in the second dimension solvent at a concentration of 6.6 g/100 ml. 3H radioactivity was detected by 2,5-diphenyloxazole fluorography at -70°C for 10 days using Kodak XR-5 film. The tyrosine marker was stained with ninhydrin as described above.
3) **RESULTS**

**A. Protein is Covalently Linked to Polio Virion RNA**

To demonstrate protein covalently linked to poliovirus RNA, $^{32}\text{P}$ labelled virion RNA was purified and digested with a mixture of ribonucleases T1, T2, and A. The digest was subjected to pH 3.5 3MM paper ionophoresis. The fractional digestion products detected by autoradiography (Fig. 1, lane 2) are the four nucleoside monophosphates and a spot (a) running toward the cathode. In a parallel experiment, virion RNA was treated with Proteinase K, repurified, and then digested with RNases T1, T2, and A. Fractionation of the products (Fig. 1, lane 1) revealed that spot (a) is protease-sensitive. Coincident with the disappearance of spot (a) is the appearance of a new spot near the position of the XC dye marker. The compound in spot (a) has been shown to contain a protein, VPg (for viral protein, genomic) linked to pUp (Flanegan, et al., 1977). The pUp is linked to VPg by its 5' phosphate; spot (a) is designated VPg-pUp. The spot running near the XC dye has been shown to be a protease K resistant oligo peptide of VPg containing covalently linked pUp (Flanegan, et al., 1977; Nomoto, et al., 1977a) and is designated "K-pUp".

**B. Purification of VPg from Poliovirus RNA**

To purify labelled VPg, infected cells were labelled with $^{32}\text{PO}_4$, virions were purified, and virion RNA was phenol-extracted. The 35S RNA was recovered from the aqueous phase and digested with ribonucleases T1, T2 and A. Such a digest should contain mainly $^{32}\text{P}$-mononucleotides and about 0.042% of the $^{32}\text{P}$ in VPg-pUp (Nomoto, et al., 1977a). The digest was then extracted again with phenol and proteins in the phenol phase were recovered by acetone precipitation. When this material was fractionated by chromatography in 0.5% sodium dodecyl sulfate on Sephadex G-25, and excluded
Virion RNA was labelled and purified as described in "Methods and Materials". A portion of the RNA was treated with protease K and then repurified by phenol extraction. RNA samples were then digested with a mixture of ribonucleases T1, T2, and A, and the products fractionated by pH 3.5 3 MM paper ionophoresis.

Lane 1: Protease K treated sample.
Lane 2: Untreated sample.
O: Origin
XC: Xylene cyanol dye marker.
Cathode is below.
peak of $^{32}\text{P}$ label was found (Fig. 2A). As previously demonstrated for VPG-pUp (Flanegan, et al., 1977), most of the excluded $^{32}\text{P}$ migrated toward the anode during pH 3.5 electrophoresis on Whatman No. 3MM paper. The included peaks from Sephadex G-25 contained a small amount of ribonucleotides contaminating the phenol layer. Thus, phenol extraction of a ribonuclease digest followed by gel filtration can rapidly purify VPG-pUp.

C. Molecular Weight Estimation for VPG-pUp

To size VPG-pUp, it was chromatographed in 0.5% sodium dodecyl sulfate on Bio-Gel A-1.5m (Fig. 2B). The majority of the $^{32}\text{P}$ migrated as a peak close to the positions of cytochrome c ($M_r = 12,500$). To further analyze the molecular weight of VPG-pUp, it was chromatographed on Bio-Gel A-5m in 6 M guanidine HCl, 0.1 M 3-mercaptoethanol (Fig. 2C). Again it appeared to be 12,000 in molecular weight. The electrophoretic mobility of VPG in the presence of sodium dodecyl sulfate would suggest a molecular weight less than 10,000 (Nomoto, et al., 1977b). The estimate of 12,000 from gel exclusion is probably a better approximation of the true molecular weight of the protein (Fish, et al., 1977).

The combination of sequential Sephadex and Bio-Gel A-1.5 chromatography of the phenol phase gave a yield of $^{32}\text{P}$ in VPG-pUp of about 0.032% of the starting material. When this was corrected by the unequal labelling of the 4 nucleotides (Flanegan, et al., 1977), it represented a recovery of about 75% of the theoretical yield.

D. VPG of Total Cytoplasmic Polio RNA

Cytoplasmic polio RNA molecules, including double-stranded RNA, and replicative intermediate RNA, contain 5' terminal protein. It was possible that some of these molecules might contain covalently bound protein larger or smaller than the VPG of virion RNA. To determine if all RNA-linked
Chromatography of VPg-pUp

$^{32}$P-labelled poliovirion RNA was digested with ribonuclease and VPg-pUp was recovered from the digest by phenol extraction and acetone precipitation as described under "Methods and Materials". A, the material recovered from the phenol was chromatographed through a column a Sephadex G-25, equilibrated with 50 mM Tris, 0.1 M NaCl, 1 mM EDTA buffer plus 0.5% sodium dodecyl sulfate (SDS) and the void volume fractions were precipitated with acetone, collected by centrifugation, dried, and redissolved in the same buffer. B, gel filtration of VPg-pUp was then carried out on a column (1.0 x 30 cm) of Bio-Gel A-1.5m equilibrated with 0.5% sodium dodecyl sulfate, 50 mM Tris, 0.1 M NaCl, 1 mM EDTA. C, VPg-pUp was chromatographed on a column of Bio-Gel A-5m in 6 M guanidine HCl, 0.1 M $\alpha$-mercaptoethanol. Blue dextran 2000 (BD), bromphenol blue (BPB), and cytochrome c (Cy) were included as visible markers. Insulin and Moloney leukemia virus p30 protein markers were detected as described under "Methods and Materials".
A. Sephadex G-25, 0.5% SDS

B. Biogel A-1.5
0.5% SDS

C. Biogel A-5
6M Guanidine

$V_0/V_0$
protein in the cytoplasm of infected cells is of homogeneous molecular weight, $^3\text{H}$ lysine labelled VPg-pUp was prepared from total cytoplasmic RNA of infected cells. Cells were labelled with $^3\text{H}$ lysine from 1.5-3.5 h post-infection in the presence of Actinomycin D. Total cytoplasmic RNA was prepared by extensive phenol extraction and the RNA was digested with ribonucleases T1, T2, and A and then re-extracted with phenol. The protein recovered from the phenol phase chromatographed exactly like VPg-pUp from virion RNA (Fig. 3). No other size class of VPg was detected.

VPg-pU and VPg labelled with $^3\text{H}$ lysine were prepared by digesting VPg-pUp (recovered from the peak fractions of Fig. 3) with bacterial alkaline phosphatase and then with venom phosphodiesterase. VPg-pU and VPg migrated at indistinguishable rates during electrophoresis through a 10-20% gradient acrylamide gel (Fig. 4, lanes 2 and 3). Both forms migrated like a protein of less than 10,000 molecular weight, as previously reported for $^{32}\text{P}$ labelled VPg-pUp (Nomoto, et al., 1977b).

When total $^3\text{H}$ lysine labelled proteins from infected cells at 3.5 h post-infection were analyzed, no protein of the mobility of VPg or VPg-pU was evident (Fig. 4, lane 1). This result indicates that either i) there is a small and undetectable pool of free VPg in the cell or ii) VPg is part of a larger polypeptide when not bound to RNA.

E. Identification of the RNA-Linked Amino Acid of VPg

Digestion of VPg-pUp with venom phosphodiesterase released pUp and digestion of VPg-pU with venom phosphodiesterase released pU (Flanegan, et al.; Nomoto, et al., 1977a). These results suggest that the linkage between VPg and the 5' terminal uridine is a phosphodiester bond. Preliminary studies (data not shown) showed that the linkage was stable under acidic conditions that would hydrolyze phosphoramidate or carboxyl-
FIGURE 3

Chromatography of $^3$H-lysine VPg-pUp on Bio-Gel A-1.5

$^3$H-lysine labelling of poliovirus proteins was done by infecting $2 \times 10^8$ HeLa cells with multiplicity of 20 with poliovirus type 1 in 50 ml minimal essential medium plus 5% fetal calf serum, 5 mg/ml actinomycin D. At 1.5 h post-infection, cells were centrifuged, washed with Earle's saline and resuspended in warmed Earle's saline plus 0.3 g/mL glutamine, 5% dialyzed fetal calf serum, 5 mg/ml actinomycin D and 5 mCi $^3$H-L lysine (60-80 Ci/mmole). Cells were harvested at 3.5 post-infection and lysed with 1% Nonidet P-40. The cytoplasm was made 0.5% sodium dodecyl sulfate and extracted twice with phenol:chloroform:isoamyl alcohol. The organic phases and interfaces were pooled and precipitated with 5 volumes of acetone. The aqueous phase was re-extracted with phenol:chloroform:isoamyl alcohol four more times to remove all protein not covalently bound to RNA. The RNA was then precipitated by the addition of 3 volumes 95% ethanol and stored at -20°C. Removal of $^3$H-lysine-labelled VPg-pUp from the RNA of infected cells and subsequent gel filtration and chromatography were performed exactly as described above for $^{32}$P-labelled VPg-pUp in Figure 2B.
FIGURE 4

Electrophoresis of VPg and Total Poliovirus-Specific Proteins

VPg-pU was converted to VPg-pU by digestion with 10 μl of 2 U/ml bacterial alkaline phosphates. An equal portion of VPg-pU was further converted to VPg by digestion with phosphatase followed by snake venom phosphodiesterase. The total 3H-lysine-labelled protein was recovered by phenol-extraction of the labelled cells used to prepare 3H-lysine-labelled VPg from RNA. VPg-pU, VPg and total labelled protein, each derived from 10^7 labelled cells, were submitted to electrophoresis through a 10-20% gradient polyacrylamide gel in sodium dodecyl sulfate and were developed by fluorography of the PPO-impregnated gel. (1) Total proteins; (2) VPg-pU; (3) VPg.
phosphate anhydride linkages. Under such conditions, however, phosphoester bonds to threonine, serine or tyrosine would be stable (Shabarova, 1970).

To begin identification of the phosphate-linked amino acid, $^{32}$P-labelled VPg-p was hydrolyzed in 2 M HCl to break all peptide bonds. The products were analyzed by pH 3.5 ionophoresis (Fig. 5A). The $^{32}$P radioactivity migrated at a peak of material (compound I) faster than either the phosphoserine or phosphothreonine markers. Treatment of this material with bacterial alkaline phosphatase released all of the radioactivity as free phosphate (Fig. 5B). Because compound I did not appear to be either phosphoserine or phosphothreonine, we investigated whether it might represent phosphate linked to tyrosine.

Poliovirus was labelled with $[^3H]$tyrosine as described under "Experimental Procedures". Purified virions were lysed by phenol extraction, and the RNA was sedimented through a sodium dodecyl sulfate-sucrose gradient. A peak of $^3$H radioactivity was recovered at 35S (Fig. 6A) co-sedimenting with $^{32}$P-labelled 35S poliovirion RNA that was analyzed on a parallel gradient. The RNA was recovered from the gradient fractions, digested with ribonucleases T1, T2, and pancreatic ribonuclease A, and chromatographed on a column of Sephadex G-25 (Fig. 6B). All of the $^3$H radioactivity appeared in the excluded volume where VPg-pUp would be expected and no radioactivity was detected in the included volume coincident with the nucleoside monophosphates. The $[^3H]$tyrosine-labelled VPg-pUp was recovered from the excluded fractions and digested with proteinase K, and the products were separated by pH 3.5 paper ionophoresis (Fig. 7A). The $^3$H radioactivity appeared as a single peak (compound II), with a mobility similar to that of the product produced by digesting $^{32}$P-labelled VPg-pUp with proteinase K (Fig. 1). The mobility of compound II was
Radioactively labelled VPg-p and VPg-pU were prepared as described under "Methods and Materials". $^{32}$P-labelled VPg-p was hydrolyzed in 2 M HCl at 110°C for 24 h and the products were analyzed before (A) and after (B) bacterial alkaline phosphatase treatment. $[^{3}H]$Tyrosine-labelled VPg-p was acid-hydrolyzed before (C) and after (D) bacterial alkaline phosphatase treatment. $[^{3}H]$Tyrosine-labelled VPg-pU was acid-hydrolyzed as above and the products were analyzed before (E) and after (F) treatment with venom phosphodiesterase. Cathode is on the left. 0, indicates position of sample application. XC marks xylene cyanol blue dye marker. Amino acid markers tyrosine (Tyr), phosphothreonine (p-Thr), and phosphoserine (p-Ser) were run in a parallel lane and detected by ninhydrin staining.
Poliovirus 35S RNA was isolated from 3H-tyrosine-labelled poliovirions by phenol extraction and sedimented through a 15-30% sucrose gradient in the presence of 0.5% sodium dodecyl sulfate. A portion of each fraction was assayed for 3H radioactivity (A). Sedimentation is from right to left. The arrow indicates the position of 32P-labelled 35S RNA in a parallel gradient. RNA was recovered from the peak and digested with ribonuclease T1, T2, and A. The digest was chromatographed on a column of Sephaex G-25 in 0.5% sodium dodecyl sulfate, and a portion of each fraction assayed for 3H radioactivity (B). BD marks the position of the excluded volume as determined by chromatographing blue dextran dye on the same column in an independent run.
35S Sucrose Gradient

\[ \text{Fraction Number} \]

400 300 200 100 0

\[ \text{Fraction Number} \]

A

B

\[ \text{Fraction Number} \]

\[ \text{Fraction Number} \]

\[ \text{Fraction Number} \]

\[ \text{Fraction Number} \]
FIGURE 7

Ionophoretic Separation at pH 3.5 of $^3$H$^\text{Tyr}$-labelled Products of Proteinase-K Digestion of VPg-pUp and VPg

$^3$H$^\text{Tyr}$-labelled VPg-pUp was purified as described under "Methods and Materials" and digested with proteinase K directly (A) and after prior treatment with venom phosphodiesterase (B). Procedures and conditions of ionophoresis and amino acid markers used were identical to those in Figure 2.
altered greatly by removal of pUp with venom phosphodiesterase prior to proteinase K digestion (Fig. 7B) indicating that in it, pUp is covalently bound to $[^3H]$tyrosine-containing material. The phosphate-free $[^3H]$tyrosine-labelled peak in Fig. 7B, however, did not co-migrate with tyrosine marker, and probably is a proteinase K-resistant oligopeptide containing one or more amino acids in addition to tyrosine (Nomoto, et al., 1977). The linkage to pUp must be through one of those amino acids.

To determine whether tyrosine was involved in the linkage, $[^3H]$tyrosine-labelled VPg-p was hydrolyzed with 2 M HCl and the products were analyzed by pH 3.5 paper ionophoresis both before (Fig. 5C) and after (Fig. 5D) bacterial alkaline phosphatase treatment. Over 90% of $[^3H]$tyrosine label was recovered in material migrating at the position of the $^{32}P$-labelled compound I shown in Fig. 5A. After phosphatase treatment, all of the $[^3H]$tyrosine label migrated like free tyrosine (Fig. 5D). The phosphate that links VPg to viral RNA therefore appears to be covalently bound to tyrosine.

To further characterize the linkage, $[^3H]$tyrosine-labelled VPg-pU was analyzed by acid hydrolysis and pH 3.5 electrophoresis exactly as above (Fig. 5E). The mobility of the major peak in Fig. 5E was unaffected by phosphatase (data not shown) yet free $[^3H]$tyrosine was generated by venom phosphodiesterase digestion (Fig. 5F). To confirm the identity of this released material, the pU was removed from $[^3H]$tyrosine-labelled VPg-pU by venom phosphodiesterase. The protein was then hydrolyzed in 2 M HCl as above, and the hydrolysate was analyzed by two-dimensional thin layer chromatography (Fig. 8). The $^3H$ radioactivity migrated as a spot coincident with the ninhydrin-stained tyrosine marker included in the
FIGURE 8

Two-Dimensional Thin Layer Chromatography

[3H]Tyrosine-labelled VPg-pU was digested with venom phosphodiesterase to remove pU and hydrolyzed in 2 M HCl, and the hydrolysate was chromatographed in two dimensions on a cellulose-coated thin layer plate. **Dimension 1**, n-butanol:acetic acid:H2O, 12:2:5. **Dimension 2**, n-butanol:pyridine:acetic acid:H2O, 32.5:29:5:20. 

3H radioactivity was detected by 2,5-diphenyloxazole fluorography. The position of unlabelled tyrosine marker was determined by ninhydrin staining and is indicated by the dashed outline.
sample. This confirms that the 5' terminal uridine of poliovirus RNA is linked to VPg by a phosphodiesterase bond to tyrosine. It also appears that the RNA-linked tyrosine is the only tyrosine residue in the VPg protein molecule, because over 90% of the [$^{3}$H]tyrosine radioactivity was phosphate-linked (Fig. 5, C and E).

4) **DISCUSSION**

Molecular weight estimation of VPg by SDS acrylamide gel electrophoresis (Nomoto, et al., 1977b; Figure 4 of this thesis) indicates that VPg is less than 10,000 molecular weight. However, VPg chromatographs as a polypeptide of approximately 12,000 molecular weight by both SDS and Guanidine-HCl denaturing solvent gel filtration chromatography. The estimation of 12,000 molecular weight for VPg from gel filtration chromatography is probably a better approximation of the true molecular weight of the protein because of the greater uncertainly in measurements of molecular weight of small proteins by SDS gel electrophoresis (Fish, et al., 1969).

Experiments described in this chapter demonstrate that VPg is bound to the 5' end of poliovirion RNA through a tyrosine residue. The acid stability of this linkage, and its sensitivity to cleavage by venom phosphodiesterase indicate that the linkage is a phosphodiester bond to the tyrosine side chain, presumably to the hydroxyl group at position 4 of the ring.

If the proposed role of VPg as a primer (Flanagan, et al., 1977) for RNA synthesis is true, the initial covalent bond formed during chain elongation would be a phosphodiester bond between the tyrosine side chain of VPg and the $\alpha$-phosphate of an activated UMP moiety probably in UTP. This could be analogous to the reaction of the *E. coli* glutamine synthetase regulatory protein PII with UTP to form 5' UMP covalently bound
to the PII protein by a phosphodiester linkage through tyrosine (Alder, et al., 1975).

Chapters IV and V describe a cellular enzyme which cleaves the tyrosine-phosphate linkage between VPg and polio RNA. If this cleavage represents the normal activity of the enzyme, then the possibility arises that the tyrosine-RNA linkage described here for poliovirion RNA is an example of a class of protein-nucleic acid linkages normally found in HeLa cells.
CHAPTER III

VPG IS LINKED TO NASCENT POLIOVIRUS RNA

1) INTRODUCTION

The finding that VPG is found on Replicative Intermediate RNA of poliovirus (Flanegan, et al.; Nomoto, et al., 1977a) led to the hypothesis that VPG may play a role in initiation of RNA synthesis. This chapter describes experiments designed to determine what proportion of nascent polio RNA molecules of various sizes contain VPG. If VPG is attached to a nascent molecule a significant time after the initiation of the RNA strand, then one might find small nascent molecules lacking VPG as the 5' end structure. Such molecules might have, for example, 5' terminal pUP, ppUP or pppUP. If VPG is added to all molecules at, or very soon after initiation, then one might find all nascent chains contain VPG. The latter model necessitates the hypothesis that VPG is removed to form mRNA. The former model does not require such an activity, since mRNA could be formed from those nascent molecules that never underwent the addition of VPG.

This chapter shows that at 3.5 h after infection, the 5' end structure of nascent polio RNA molecules is VPG. No nascent chains with 5' terminal pUP were detected, consistent with the hypothesis that polio mRNA is formed by the enzymatic removal of VPG from RNA.

2) MATERIALS AND METHODS

A. Fractionation of Nascent Chains From RI RNA

RI RNA, which had been recovered from the excluded volume of a Sepharose 2B column (Baltimore, 1968) was precipitated with 2.5 volumes of ethanol at -20°C in the presence of 0.3 M sodium acetate. The precipitate was collected by centrifugation and dissolved in distilled water.
treated with 0.01% diethylpyrocarbonate, and sodium dodecyl sulfate was added to 0.5%. In some experiments unlabelled virion 35S RNA (final concentration, 50-100$\text{ME}$/ml) was included. The RNA was then heated at 75°C for 5 min (Baltimore, 1968) and quickly cooled on ice, and glycerol was added to a final concentration of 8%. The sample, usually 0.5 to 1.0 ml, was layered on a Sepharose 2B column (1.6 x 54 cm) (Baltimore, 1968), and the RNA was eluted with a solution containing 0.1 M NaCl, 0.05 M Tris-hydrochloride (pH 7.5), 0.001 M EDTA, and 0.5% sodium dodecyl sulfate. Fractions (1 ml) were collected and assayed for Cerenkov radiation. The RNA in the fractions to be analyzed was precipitated with ethanol and stored at -20°C. Size calibrations of the column were done by chromatographing $^{32}$P-labelled Uukuniemi virus RNA segments under identical conditions. The three RNA segments have apparent molecular weights of 2.4 x $10^6$ (L RNA), 1.1 x $10^6$ (M RNA), and 0.5 x $10^6$ (S RNA) (Pettersson, et al., 1977). A linear relationship was observed between the logarithm of the molecular weights and the corresponding elution position.

B. Sodium Dodecyl Sulfate-Polyacrylamide Gel Analysis

The protein covalently linked to poliovirus RNA species was prepared as described in Chapter II. Briefly, labelled RNA was digested to mononucleotides with a mixture of RNases T1, T2, and A, followed by phenol extraction. The protein was then precipitated from the phenol phase with 9 volumes of acetone at -20°C. Bovine serum albumin (10$\text{mg}$) and cytochrome c (about 5$\text{mg}$) were included as carriers throughout the procedure. The precipitated protein was collected by centrifugation, dried in vacuo, taken up in sample buffer, boiled for 2 min, and analyzed on a 10 to 20% gradient slab polyacrylamide gel (Laemmli, 1970). The gels were fixed, stained, destained, and dried, and an autoradiograph was prepared at 70°C.
by using Kodak XR-5 films and an intensifying screen. This procedure regularly resulted in a rather broad VPg band, probably due to some acetone-precipitable residue in the phenol. The radioactive bands were cut out from the gel, and the radioactivity was quantitated either by counting Cerenkov radiation or in a toluene-based scintillation fluid (Econofluor; New England Nuclear Corp.). In each experiment, the efficiency of recovery of VPg was monitored by measuring the recovery of VPg from a known amount of virion RNA. When the unequal labelling of the different mononucleotides is considered, the theoretical yield of radioactivity in protein-pUp is 0.042% of the total (Flanegan, et al., 1977). Usually 0.015 to 0.022% was observed, giving a recovery of VPg of 25 to 50%.

C. Base Composition Analysis

RNA to be analyzed was completely digested with RNases T1, T2, and A. In some cases the digest was extracted with phenol:chloroform:isoamyl alcohol (25:24:1) and the mononucleotides precipitated from the aqueous phase with 9 vol of acetone at -20°C. The samples were then subjected to pH 3.5 ionophoresis on Whatman 3 MM paper (Barrell, 1971). The radioactive spots were visualized by autoradiography using Kodak XR-5 or no-screen films. The spots were cut out from the paper and quantitated either by counting Cerenkov radiation or in Econofluor using a Beckman LS-330 scintillator.

3) RESULTS

A. Isolation and Fractionation of Nascent Chains From RI RNA

Poliovirus RI RNA labelled with $^{32}$P was extracted from the cytoplasm of infected HeLa cells at 3.5 h after infection, a time at which the maximum rate of viral RNA synthesis occurs (Baltimore, et al., 1966).
Labelled RNA was precipitated with 2 M LiCl, phenol extracted, and chromatographed on Sepharose 2B (Fig. 9A); as previously determined, the material eluting in the excluded volume consists of more than 95% pure RI RNA (Baltimore, 1968). The included material, mainly located in fractions 50 to 60, consisted of poliovirus 35S RNA as shown by sucrose gradient centrifugation analysis (data not shown). To release nascent chains from their minus-strand template, RI RNA was heated at 75°C for 5 min, quickly cooled, and immediately rechromatographed on Sepharose 2B (Fig. 9B) (Baltimore, 1968). This treatment released 40 to 50% of the radioactivity as RNA that eluted as a much broader peak than 35S RNA; as previously shown, this RNA consists mainly of newly synthesized RNA of plus polarity (Baltimore, 1968).

To confirm that the released material represented nascent chains and that they were fractionated according to their size, portions of the included broad peak of Fig. 9B were submitted to fingerprint analysis on two-dimensional polyacrylamide gels. If fractionation of nascent chains had occurred, then RNase T1-derived oligonucleotides from the 3' end of the RNA should have become gradually depleted as the nascent chains became shorter. Fractions from the column were combined into 15 pools (Fig. 9B). The fingerprint analysis of these pools was carried out by Ralf Pettersson (Pettersson, et al., 1978). The results, (not shown here), confirmed that the released material in Fig. 9B was almost exclusively plus strand sequences, and that pools of decreasing size contained RNA of decreasing complexity, consistent with their being nascent chain populations fractionated by size.

When L (2.4 x 10^6 daltons), M (1.1 x 10^6 daltons), and S (0.5 x 10^6 daltons) RNA segments from Uukuniemi virus (a bunyavirus) (Pettersson,
FIGURE 9
Fractionation of Nascent Chains From Poliovirus RI RNA

(A) Poliovirus RI RNA labelled with $^{32}$P was extracted from HeLa cells at 3.5 h after infection and precipitated with 2 M LiCl at -20°C. The precipitate was phenol extracted, and the RNA was precipitated with ethanol. The RI RNA was freed of $^{35}$S RNA by chromatography on Sepharose 2B. (B) The RNA excluded from the column was pooled and ethanol precipitated. The nascent chains were released from their template by heating at 75°C for 5 min and fractionated on Sepharose 2B. In both cases 1-ml fractions were collected and assayed for radioactivity by taking 5-μl fractions for Cerenkov radiation. Fractions containing released nascent chains were pooled as indicated to give 15 3-ml pools. Uukuniemi virus L, M, and A RNA segments labelled with $^{32}$P were used in separate runs as known molecular weight markers.
et al., 1977) were used to calibrate the agarose column, RNA eluting in pools 1 to 3 consisted mainly of full-sized poliovirus RNA ($2.4 \times 10^6$ daltons), whereas the RNA in pools 14 and 15 had a molecular weight of about 120,000 to 190,000.

B. **Analysis of the 5' End of Nascent Chains**

A small protein (VPg) is linked to a pUp residue at the 5' end of poliovirion RNA. VPg is also found associated with purified RI RNA (Flanegan, et al., 1977). To investigate whether the protein could be recovered from nascent chains of different lengths, pools 1 to 15 (Fig. 9B) were digested with a mixture of RNases T1, T2, and A. This treatment degrades the RNA to 3'-mononucleotides, leaving the protein covalently linked to the pUp residue (Flanegan, et al., 1977), and, therefore, the protein can be detected by its content of labelled phosphate. As described in Chapter II, the digested RNA was phenol extracted, and the material which was extracted into the phenol phase was precipitated with acetone. The precipitate was then analyzed by polyacrylamide gel electrophoresis (Fig. 10). Two samples of $^{32}$P labelled virion RNA treated similarly were used as controls for the recovery of VPg-pUp from each pool. A protein migrating with the same mobility as VPg was recovered. No other species of radioactively labelled protein was detected except in the marker lanes derived from virions; the virion contaminants do not purify with 35S RNA. The variable radioactivity that did not enter the gel (Fig. 10) has not been investigated. Calculation of the ratio of the relative molar yield of VPg-pUp to that of the nascent chains in each pool showed that in pools 1 to 7, approximately one mole of VPg-pUp was recovered per mole of RNA chains (Table 1). In pools 8 to 15 a decreased recovery of 0.3 to 0.6 was observed. Accurate estimation of the ratio in the later pools,
RNA from pools 1-15 (Fig. 9) was digested with RNases T1, T2, and A and phenol extracted. The protein from the phenol phase was precipitated with acetone and analyzed by electrophoresis through a 10 to 20% gradient slab gel in sodium dodecyl sulfate. Two samples of virion RNA were used as controls (left and right lanes, marked V). The gel was dried, and an autoradiogram was prepared. BPB, Bromophenol blue marker.
NOTES TO TABLE 1

a) Calculated as $\frac{CPM}{\text{chain length}}$ and normalized to pools 1-3, which contained full-sized virion RNA. VPg was assumed to be linked to pUp.

b) $\frac{\text{Relative molar yield of VPg}}{\text{Relative molar yield of nascent chains}}$

c) Pools 1, 2 and 3 contained full-sized poliovirion RNA and the mean yields of chains and VPg in these pools were therefore taken as one.
<table>
<thead>
<tr>
<th>Pool Number</th>
<th>CPM in RNA x 10^-3</th>
<th>Average Chain Length</th>
<th>Relative Molar Yield of Chains a)</th>
<th>CPM in VPg</th>
<th>Relative Molar Yield of VPg a)</th>
<th>VPg Chains b)</th>
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<tr>
<td>1</td>
<td>405</td>
<td>7,500</td>
<td>1.0 c)</td>
<td>48</td>
<td>1.0 c)</td>
<td>1.0</td>
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<tr>
<td>2</td>
<td>628</td>
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<tr>
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<td>4.4</td>
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<td>3.1</td>
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</tr>
<tr>
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</tr>
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</table>
RNA from pools 1 to 15 (Fig. 9) was digested with RNases T1, T2, and A and phenol extracted. The aqueous phase was precipitated with acetone, dried, redissolved in water and subjected to 3 MM paper ionophoresis as described in "Methods and Materials". $^{32}\text{P}$ radioactivity was detected by autoradiography. XC, xylene cyanol dye marker. 0, origin. Cathode is below. The relative mobilities of phosphate (Pi) and pU with respect to the four nucleoside monophosphates was determined on a parallel ionophoretic run.
To study whether any other kind of 5' end, such as pUp was present on the nascent chains, RNA from each pool was again digested to 3' monophosphates by RNases T1 T2 and A, and subjected to pH 3.5 ionophoresis on Whatman 3 MM paper. Apart from some material (<3%) left at the origin, and some inorganic phosphate, no material other than the four 3' monophosphates could be detected (Fig. 11).

4) DISCUSSION

A protein of the same size and electrophoretic mobility as the one on poliovirion RNA is covalently linked to the 5' end of nascent chains of poliovirus RI. VPg was found to be linked to nascent plus chains of all lengths. The ratio of relative molar yield of VPg to that of nascent chains was close to 1:1 for chain lengths between 7500-2500 nucleotides. Thereafter (pools 8-15, Fig. 9: Table 1), the ratio varied between 0.3 and 0.6. Accurate estimation of the ratio of VPg to chains shorter than about 2500 nucleotides was difficult because: (i) no size marker shorter than 0.5x10^6 daltons was used to show that a linear relationship between the logarithm of the molecular weights and elution position actually existed for the short RNA; (ii) the size fractionation for short chains may not have been as good as for larger RNA molecules, especially since trailing of large molecules into fractions with small molecules occurred; (iii) some degradation of RNA may have occurred, resulting in the recovery of short molecules with 5' ends generated by RNA breakage.

The RNA analysed in these experiments was phenol extracted prior to ribonuclease digestion. This fact leaves open the possibility that there existed a class of short nascent chains covalently bound to a protein larger than VPg. These could have been partitioned to the phenol phase during
extraction, and such larger proteins might not have been detected in this experiment. Reconstruction experiments (data not shown) determined that VPg alone is not sufficient to render phenol soluble oligonucleotides longer than approximately 12 nucleotides.

No pUp was detected at the 5' ends of nascent poliovirus RNA molecules. In the nascent chain pool with the poorest yield of VPg (pool 15, with 0.3 VPg/chain) 31 cpm $^{32}$P VPg were detected. If the remaining 70% of nascent chains in this pool had pUp, then one would expect approximately 70 cpm in pUp. The minimum sensitivity of the autoradiographic procedure employed in figure 11 was on the order of 5-10 cpm. Therefore, less than approximately 10% of nascent chains in pool 15 had 5' terminal pUp. Other 5' end structures not separable from the nucleoside 3' monophosphates by pH 3.5 3MM paper ionophoresis would not be detected in this experiment. Therefore, although these experiments show that VPg is attached to the majority of nascent polio RNA chains, it is still not known how or when VPg becomes attached to each strand. These experiments do not rule out the possibility that 5' end structures other than VPg or pUp might be on some nascent molecules, and were not detected for reasons outlined above.
Poliovirus mRNA isolated from polyribosomes lacks VPg, but its 5' nucleotide sequence is identical to that of virion RNA (Pettersson, Hewlett and Baltimore, 1977). The recovery of VPg from nascent RNA molecules implies, as suggested previously, (Pettersson, Hewlett and Baltimore, 1977) that VPg must be cleaved from a fraction of newly-made RNA strands to give rise to the 5'-pUp found on polyribosomal RNA. Because no pUp could be demonstrated on the nascent chains, cleavage apparently does not take place while the RNA is still associated with the RI.

Pettersson et al., (1978), reported the recovery of VPg from the poly-U tract isolated from the negative strands of the RI and double-stranded polio RNA's. No other species of 32P-labelled protein could be extracted from any virus-specific RNA's. It is therefore likely that the same species of protein is linked to virion RNA, nascent plus-strands, and minus-strands.

Nomoto et al., (1977a), have also reported the isolation of a protein from the poly-U of poliovirus RI and double-stranded RNA after treatment with RNAses T1, T2, and A. They showed that pUp could be released from the protein after digestion with venom phosphodiesterase. This indicates that the linkage between the protein and the poly-U may be the same as that to the virion RNA.
CHAPTER IV
AN ENZYMATIC ACTIVITY IN UNINFECTED CELLS THAT
CLEAVES THE LINKAGE BETWEEN POLIOVIRION RNA AND
THE 5' TERMINAL PROTEIN

1) INTRODUCTION

The single-stranded RNA genome of poliovirus contains a protein (VPg) covalently bound to its 5' terminal phosphate (Flanegan et al., 1977; Nomoto et al., 1977a). VPg has a molecular weight of approximately 12,000 daltons and contains one tyrosine residue that is linked by a phosphodiester bond to the RNA molecule (Ambros and Baltimore, 1978). VPg is found on negative-strand viral RNA and on nascent poliovirus RNA molecules (Pettersson, Ambros and Baltimore, 1978). Poliovirus messenger RNA, however, lacks the 5' terminal protein and instead terminates with a 5' phosphate followed by the same nonanucleotide sequence that is 5' terminal in virion RNA (UUAAAACAG) (Nomoto et al., 1977b; Pettersson et al., 1977). Since VPg is the only 5' terminal structure detectable on nascent poliovirus RNA, it appears that the protein-RNA bond has been cleaved in RNA destined to act as viral messenger RNA. To investigate this cleavage reaction, we have assayed several cell-free extracts for an activity that breaks the tyrosine-phosphate bond between VPg and poliovirus RNA. This chapter demonstrates that such an enzymatic activity exists in extracts of both poliovirus-infected and uninfected HeLa cells, as well as in cell-free extracts of rabbit reticulocytes, mouse L cells and wheat germ. The activity specifically cleaves the tyrosine-phosphate bond.
Partially purified preparations of this activity (subsequently referred to as unlinking activity, or unlinking enzyme) from both the cytoplasmic fraction of HeLa cells and from washed HeLa nuclei remove VPg leaving the protein intact and the RNA only partially degraded. The most pure fraction removes VPg leaving infectious protein-free RNA.

2) METHODS AND MATERIALS

Enzymatic Removal of VPg from Poliovirus RNA by a HeLa Enzyme

A. Preparation of Substrates

$^{3}$H-tyrosine-labelled poliovirion RNA was prepared as described in Chapter II and stored at -70°C in 0.1 mM EDTA pH 7.5 at a concentration of 0.2 pmole/ml = 0.5 mg/ml. When $8 \times 10^8$ polio infected HeLa cells were labelled with 5 mCi $^{3}$H-tyrosine, specific activity of purified virion RNA was approximately $1000^{3}$H cpm/mg.

$^{32}$P-labelled 5' terminal T1-resistant oligonucleotide of poliovirion RNA was purified by cellulose acetate electrophoresis as described elsewhere (Flanegan et al., 1977) and recovered by elution from cellulose acetate with 1% SDS, followed by ethanol precipitation with carrier yeast tRNA. The oligonucleotide was stored at -70°C in 0.1 mM EDTA, pH 7.5 at 0.2 pmole oligonucleotide/ml.

B. Cell Extracts

Reticulocyte lysates, L cell and wheat germ cell-free extracts were gifts from John K. Rose. Wheat germ extracts were prepared according to a method described elsewhere (Roberts and Paterson, 1976). Reticulocyte lysates, HeLa and L cell cytoplasmic extracts were prepared as described elsewhere (Villa-Komaroff et al., 1974), with some
modifications (Rose et al., 1978).

C. Nuclear Wash

An extract of HeLa cell nuclei was made as follows: approximately 4 x 10⁸ cells were washed twice with Earle’s saline and resuspended in a volume of 10 mM HEPES (pH 7.5), 15 mM KCl, 1.5 mM Mg acetate, 6 mM 2-mercaptoethanol (hypotonic buffer) equal to about twice the packed cell volume. After 5 min at 0°C, the cells were broken with 15 strokes of a Dounce homogenizer. Nuclei plus larger cell debris were recovered by centrifugation at 5000 rpm for 2 min at 0°C in a Sorvall type SS-34 rotor. The sediment was washed twice by resuspension in hypotonic buffer followed by centrifugation for 2 min at 5000 rpm. It was then resuspended in 10 mM HEPES (pH 7.5), 1.5 mM Mg acetate, 6 mM 2-mercaptoethanol, 200 mM KCl, and allowed to stand on ice for 45 min with occasional gentle mixing. The supernatant was then recovered by centrifugation at 20,000 x g for 30 min. This nuclear wash was stored at -70°C in small portions.

D. Incubation Conditions for Removal of VPg from Poliovirus RNA

All incubations were performed in 1.5 ml polypropylene Eppendorf microcentrifuge vials. To each vial was added a mixture of 15 µl 10 mM Tris pH 7.5, 1.5 mM MgCl₂, 5 mM β-mercaptoethanol, 10% glycerol, (TMMG buffer) and 0.5 µl polio RNA substrate in 0.1 mM EDTA, pH 7.5, (approximately 250 cpm in 0.1 pmole RNA). 5 µl of extract or TMMG buffer were then added and the mixture was mixed briefly and left to incubate at 30°C or 37°C. At the end of the incubation time the degree of removal of VPg from the RNA was immediately measured.
E. Assay for Removal of VPg from the 5'-Terminal Ti Oligonucleotide of Poliovirus

When the $^3$2P-labelled 5' terminal nonanucleotide of poliovirus was used as a substrate, removal of the VPg attached to it was detected by phenol extraction. The nonanucleotide with VPg is phenol-soluble, whereas with VPg removed, it is partitioned to the aqueous phase. After incubation, the sample volume was adjusted to 80 $\mu$l with water and extracted with 80 $\mu$l of redistilled phenol. The aqueous phase was removed and the radioactivity in each phase was measured by liquid scintillation counting in Aquasol or by Cerenkov radiation. The degree of removal of the protein from the nonanucleotide was expressed as the ratio of radioactivity in the aqueous phase to the total radioactivity in both phases. In some cases, the phenol phase of the control sample and the aqueous phase of the extract treated sample were precipitated at -20°C with 10 vol of 95% ethanol in the presence of 0.4 M NaCl and 20 $\mu$g yeast-carrier tRNA. The precipitates were recovered by centrifugation and redissolved in H$_2$O, and a portion of each was analyzed by two-dimensional electrophoresis and homochromatography (Pettersson, et al., 1977).

F. Gel Filtration Assay for the Removal of VPg from $^3$H-Tyrosine-labelled Poliovirion RNA

When $^3$H-tyrosine-labelled virion RNA was used as a substrate, removal of the protein was detected by Biogel A1.5 M column chromatography. In the presence of sodium dodecylsulfate, VPg free of RNA elutes from this column at the position of a protein of about 12,000 molecular weight (Ambros and Baltimore, 1978). VPg attached to RNA elutes with the excluded volume of the column. $^{32}$P-labelled 35S poliovirion RNA was
included in each sample to monitor ribonuclease activity. After incubation in the cell extract, 20 μl of 30% sucrose, 0.5% sodium dodecyl sulfate, 50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA and 10 μg Bovine serum albumen were added, and the sample was mixed thoroughly and layered onto a 1 x 10 cm Biogel A1.5 M column. The column was equilibrated with 0.5% sodium dodecyl sulfate, 50 mM Tris (pH 7.5), 0.1 M NaCl, 1 mM EDTA, and eluted in 15 drop fractions with the same buffer. Radioactivity was measured by liquid scintillation counting in Aquasol. Counter settings were adjusted so that 32P and 3H radioactivity was measured with no significant spillover. The degree of removal of VPg from RNA was expressed as the ratio of 3H radioactivity included in the column fractions containing proteins of 12,000 molecular weight or less to the total 3H radioactivity eluted from the column.

G. Phenol Extraction Assay for the Removal of VPg from 3H-Tyrosine-labelled Poliovirion RNA

Removal of 3H-tyrosine-labelled VPg from poliovirus 35S RNA was also assayed by phenol extraction exactly as described above for the 32P-labelled 5' terminal nonanucleotide substrate. When attached to 35S RNA, VPg remains in the aqueous phase after phenol extraction; freed from RNA, it partitions to the phenol phase. The portion of VPg removed was expressed as percentage of 3H radioactivity which was phenol-soluble.

When many samples or fractions were assayed at once, the following procedure was used: Identical 15 μl samples of TMMG buffer containing 0.1 pmole (approximately 250 cpm) 3H-tyrosine-labelled polio 35S RNA substrate were transferred to individual 1.5 ml polypropylene Eppendorf vials. 5 μl of TMMG buffer was added to one tube (control), and to each
of the rest was added 5 μl of a dilution of the fraction to be assayed. All tubes were incubated at 30°C for 30 min and then diluted and phenol extracted as described above. The entire aqueous phase of each sample was transferred to a scintillation vial and 3H radioactivity measured in Aquasol or Bray's solution. Unlinking activity was scored as decrease in radioactivity in the aqueous phase relative to the control sample aqueous phase. These data were converted to pmole 3H VPg released from RNA based on the measured specific activity of the 3H-tyrosine-labelled 35S RNA substrate, and the assumption of one VPg molecule per RNA molecule.

### H. K-Peptide Test for Cleavage of the Tyrosine-Phosphate Bond of Poliovirus RNA

The only tyrosine-containing protease K-resistant peptide of VPg, called K-peptide, is the one involved in the linkage to pUp (Chapter II). When the linkage is intact, treatment of 3H-tyrosine-labelled 35S polio RNA with a mixture of RNases T1, T2, and A, followed by protease-K leaves 3H-labelled K-pUp. When the linkage is broken, free K-peptide is produced by protease-K. These two structures can be resolved by pH 3.5 3 MM paper ionophoresis (see Fig. 7). Therefore, a definitive test for the cleavage of the tyrosine-phosphate bond employs the following protocol: 3H-tyrosine-labelled poliovirus RNA is treated with the enzyme fraction to be assayed, under standard conditions. At the end of the incubation time, a mixture of ribonuclease T1, T2, and A, is added and digestion is continued at 37°C for 1 h. Protease-K is then added to a final concentration of 1 mg/ml and the sample is further incubated at 37°C for 1 h. Samples are then fractionated by pH 3.5 3 MM paper ionophoresis. The paper is then dried, lanes fractionated and
radioactivity measured as described in Chapter II.

I. Glycerol Gradient Centrifugation

A 200 μl sample of a HeLa cell nuclear wash was sedimented through a 15-30% glycerol gradient containing 10 mM Tris (pH 7.5), 50 mM KCl, 1.5 mM MgCl₂, 5 mM β-mercaptoethanol in a Beckman SW 41 rotor at 40,000 rpm for 51 h at 4°C. Samples were collected and a 5 μl portion of each was assayed for unlinking activity using ³H-tyrosine-labelled 35S poliovirion RNA as substrate. Unlinking of VPg from RNA was measured by phenol extraction. Bovine serum albumen (4.4S) and cytochrome-c (1.7S) markers were sedimented in a parallel gradient.

J. S-100 Fractions of HeLa Cells

For purification of HeLa unlinking activity, the starting fractions were prepared differently than the extracts described above.

4 x 10⁹ HeLa cells (4 x 10⁵ cells/ml) were centrifuged at 1200 rpm for 5', washed twice with RSB (10 mM Tris, 10 mM NaCl, pH 7.5) by repeated centrifugation, and the pellet was resuspended in 20 ml of hypotonic buffer (10 mM HEPES, pH 7.5, 15 mM KCl, 1.5 mM Mg Acetate, 6 mM 2-mercaptoethanol). The cells were allowed to swell at 0°C for 5 min, then were broken with 15 strokes of a dounce homogenizer. Nuclear plus large cell debris were recovered by centrifugation at 5000 rpm for 2 min in a Sorvall type ss-34 rotor at 4°C. The cytoplasmic supernatant was stored at 0°C. The nuclear pellet was washed by resuspension in TMMG buffer followed immediately by a 2 min centrifugation at 5000 rpm in the Sorvall. The supernatant was discarded and the nuclei were resuspended in 25 ml of TMMG plus 200 mM KCl and were incubated at 0°C for 45 min with occasional gentle mixing. Both the nuclear suspension and cytoplasmic supernatant were then centrifuged at 10,000 rpm for 20 min.
at 4°C. The supernatants were then collected and centrifuged at 100,000 g for 1 h in a Beckman SW 41 rotor at 4°C. The nuclear wash S-100 supernatant, and cytoplasmic S-100 supernatant were then dialysed against TMMG plus 50 mM KCl. Any precipitate which formed during dialysis was removed by centrifugation at 10,000 rpm for 10 min at 4°C. These supernatant fractions, designated nuclear S-100 and cytoplasmic S-100, were stored in a 4°C refrigerator in an ice bath.

K. DEAE-Sephadex Chromatography of HeLa Unlinking Activity

A 1.5 x 15 cm column of DEAE-Sephadex A-25 was poured and equilibrated with TMMG + 50 mM KCl. Approximately 25 ml of sample (Nuclear S-100 or cytoplasmic S-100) was applied to the column, and the column was washed with TMMG, 50 mM KCl until the OD$_{280}$ (as monitored by an LKB Uvicord II spectrophotometer) returned to baseline value. The column was then eluted with a 150 ml 50 mM-200 mM KCl gradient (flow rate = 10-20 ml/h). 5 ml fractions were collected and 5 μl samples were assayed for unlinking activity using $^3$H-tyrosine-labelled polio RNA as substrate. Peak fractions were pooled and stored without dialysis.

L. Cibacron Blue-Sepharose Chromatography of HeLa Unlinking Enzyme

Cibacron Blue F3GA dye coupled directly to Sepharose 2B (Reyes and Sandquist, 1978) was a gift from Dr. Pim Zabel.

The pooled DEAE-Sephadex peak of unlinking activity was adjusted to 150 mM KCl and was applied to a 1 x 1.5 cm column of Cibacron Blue-Sepharose equilibrated with 150 mM KCl in TMMG buffer. OD$_{280}$ was monitored as above and when the column was washed free of unbound protein, bound material was eluted with a 30 ml gradient from 150 mM-1000 mM KCl in TMMG buffer. 1 ml fractions were collected. A 2 μl sample of each fraction was assayed for unlinking activity using the $^3$H-tyrosine-labelled
35S RNA substrate. Removal of VPg from RNA was measured by phenol extraction. The peak fractions of activity, eluting at 400-600 mM KCl, were pooled and dialyzed for 12 h at 0°C against 10 mM Tris, 1.5 mM MgCl₂, 5 mM β-mercaptoethanol, 50% glycerol, pH 7.5. This dialyzed "Cibacron pool" was aliquotted and stored in 400μl portions at -70°C. A working stock was removed from -70°C and kept at -20°C. The half-life of unlinking activity stored this way is about 2 months at -70°C in 50% glycerol, and approximately 2-3 weeks at -20°C in 50% glycerol. The DEAE-Sephadex pooled fractions are stable in 10% glycerol at 0°C for up to 1 week. Generally, however, the cibacron blue-sepharose column was run immediately after collecting and assaying the DEAE-Sephadex fractions.

DEAE cellulose was found to be an unsatisfactory material for ion exchange chromatography of HeLa unlinking enzyme. Some substance eluting from the cellulose matrix seems to irreversibly inhibit the enzyme. DEAE-Sephadex has presented no such problem.

Protein concentration in the enzyme fractions was measured by the Lowry (1951) method.

N. Agarose Gel Electrophoresis

1% agarose tube gel electrophoresis of poliovirus RNA was performed as described elsewhere (Hewlett, et al., 1976).

N. Polio RNA Infectivity Assay

Polio RNA specific infectivity was measured using the assay described by Spector and Baltimore (1974) with one variation. Dilutions of transfected cells were mixed with indicator cells in DME and added to 60 mM tissue culture dishes. The cells were allowed to attach for 30 min at 37°C. The supernatant was removed, and the cells were overlayed with 1% agar in DME + 5% fetal calf serum. Plates were placed at 37°C in a CO₂ incubator
and plaques were counted 48 h later.

3) RESULTS

A. Release of VPg from Poliovirion RNA in HeLa Cell Extracts

To assay the removal of VPg from poliovirion RNA, gel exclusion chromatography was used. $^3$H-tyrosine-labelled VPg in poliovirion RNA was excluded from a Biogel A1.5 M column in sodium dodecyl sulfate, as was the $^{32}$P-labelled poliovirus RNA marker (Fig. 12A). Extensive ribonuclease digestion of the RNA caused the $^3$H-tyrosine label to elute coincidently with cytochrome-c in agreement with the previous demonstration that free VPg has a molecular weight of 12,000 daltons (Chapter II; Ambros and Baltimore, 1978); the $^{32}$P mononucleotides were totally included in the column (Fig. 12B). When $^3$H-tyrosine-labelled VPg was deliberately degraded with proteinase K while still attached to the RNA, $^3$H-tyrosine radioactivity remained associated with the RNA (Fig. 12C). This result is in agreement with the previous finding that a tyrosine-phosphate bond links VPg to poliovirus RNA (Chapter II; Ambros and Baltimore, 1978).

When $^3$H-tyrosine-labelled poliovirion RNA was incubated in a cytoplasmic extract from HeLa cells, after 15 min almost 90% of the $^3$H radioactivity was in very low molecular weight material, eluting with the included fractions of the Biogel A1.5 M column (Fig. 12D). The $^{32}$P marker RNA was degraded somewhat, but not sufficiently to account for the small size of the $^3$H-tyrosine-containing material. Because the bond between the tyrosine residue in VPg and the poliovirus RNA 5' terminal phosphate is protease-resistant (Chapter II; Ambros and Baltimore, 1978), the result shown in Figure 12D, implies that HeLa cell cytoplasm
FIGURE 12

Biogel A1.5 M Column Chromatography of
Poliovirus RNA After Incubation in Extracts of HeLa Cells

Samples containing a mixture of approximately 200 cpm each of $^{32}$P and $^3$H-tyrosine-labelled $^{35}$S poliovirus RNA were incubated under each of the conditions described below, followed by gel filtration chromatography as described in "Methods and Materials". Fractions were collected and $^{32}$P (○—○) and $^3$H (●—●) radioactivity was measured. Incubation conditions were (A) buffer for 15 min at 37°C; (B) a mixture of ribonucleases T1, T2, and A for 15 min at 37°C; (C) 500 μg/ml proteinase-K for 1 h at 37°C; (D) HeLa cytoplasmic extract for 15 min at 37°C. Preparation of extracts is described in "Methods and Materials". The column was calibrated by chromatographing a sample containing blue dextran 2000 (BD), cytochrome(Cy) and bromophenol blue (BPB). Material chromatographed in lane (E) had been incubated in a heLa nuclear extract for 15 min at 37°C.
contains an enzyme able to cleave the VPg-RNA linkage. VPg appears to be degraded by this extract because the $^{3}$H radioactivity is in material smaller than the 12,000 molecular weight size of intact VPg. Incubation of $^{3}$H-tyrosine-labelled poliovirus RNA in a nuclear wash from HeLa cells also removed the $^{3}$H from the RNA but left most of the $^{3}$H chromatographing at the position of cytochrome-c (Fig. 12E). As was the case with the cytoplasmic extract, the nuclear wash did not degrade the $^{32}$P marker RNA sufficiently to account for the size of the $^{3}$H-tyrosine-containing material.

B. The Tyrosine-Phosphate Bond is Cleaved in Cell Extracts

The HeLa nuclear wash appears to contain an activity able to unlink VPg from poliovirus RNA while leaving both the RNA and VPg relatively intact. To confirm that the $^{3}$H-tyrosine-containing material that was released was actually VPg free of RNA, a limit protease digestion was performed. For marker, a control sample of $^{3}$H-tyrosine-labelled VPg, freed of covalently bound RNA by venom phosphodiesterase digestion of VPg-pUp (Flanegan et al., 1977), was digested with proteinase K, and the products were analyzed by pH 3.5 3 MM paper ionophoresis. All the $^{3}$H radioactivity in the control sample was found in a peak which we have designated oligopeptide K (Fig. 13A; see also Fig. 7B). The mobility, relative to the XC dye of oligopeptide K covalently bound to either phosphate (K-p), uridine 5'-monophosphate (K-pU) or pUp (K-pUp), was established in parallel ionophoretic analyses. $^{3}$H-tyrosine-containing material with mobility identical to oligopeptide K was generated by proteinase K digestion of the sample of $^{35}$S virion RNA that had been incubated in a HeLa nuclear wash (Fig. 13B). This result further demonstrates that HeLa cells contain an activity capable of breaking the bond between
FIGURE 13
Ionophoretic Separation at pH 3.5 of $^3$H-Tyrosine-Labelled Products of Proteinase K Digestion of VPg Unlinked From RNA

(A) $^3$H-tyrosine-containing K-resistant oligopeptide (K) was prepared by proteinase-K digestion of $^3$H-tyrosine-labelled VPg, derived from VPg-pUp by venom phosphodiesterase digestion. The proteinase-K digest was separated by pH 3.5 3 M paper ionophoresis, and the paper was fractionated and analyzed for $^3$H radioactivity. K-peptide covalently bound to phosphate, pU or pUp (K-p, K-pU, K-pUp) was made by proteinase-K digestion of VPg-p, VPg-pU or VPg-pUp (Ambrose and Baltimore, 1978), respectively, and the products were analyzed in identical ionophoretic analyses.

(B) $^3$H-tyrosine-labelled 35S RNA was incubated with HeLa nuclear wash under the same conditions described in Figure 12D; followed by proteinase-K digestion. The products were analyzed as described above. XC dye and tyrosine markers were included in a parallel lane. Tyrosine was detected by ninhydrin stain. (o) indicates the position of sample application. Cathode is on the left.
the tyrosine in VPg and the 5' terminal phosphate of poliovirus RNA.

C. Cleavage of VPg from the 5' Terminal Oligonucleotide of Poliovirus RNA

To confirm the observation that removal of VPg from poliovirus RNA does not involve cleavage of any phosphodiester bonds between ribonucleotides near the 5' end of the RNA, the 5' terminal nonanucleotide was purified from $^{32}$P-labelled poliovirion RNA and analyzed by two-dimensional electrophoresis and homochromatography. The nonanucleotide with VPg attached migrated slowly during cellulose acetate electrophoresis (first dimension) but rapidly in the homochromatography dimension (Fig. 14A). After incubation of the nonanucleotide in a HeLa cell extract to remove VPg, all of the $^{32}$P radioactivity migrated as a discrete species found at a position on the fingerprint markedly different from that of the nonanucleotide with VPg attached (Fig. 14B). The migration of this oligonucleotide was similar to that of the 5' terminal nonanucleotide of poliovirus messenger RNA (pUUAAAACAG) (Pettersson et al., 1977). To analyze the composition of the species contained in the major spots in Figures 14A and 14B, a portion of each was digested with a mixture of ribonucleases T1, T2, and A. This treatment generates 3' monophosphates and releases any 5' terminal structure. The products were analyzed by pH 3.5 paper ionophoresis. Nuclease digestion of the nonanucleotide with VPg attached (control) released Up plus VPg-pUp but not pUp (Fig. 15A); digestion of the nonanucleotide unlinked from VPg released pUp as well as Up (Fig. 15B). The compositions of the two oligonucleotides were otherwise identical (Table 2), and are consistent with the published sequence of the 5' end of poliovirus RNA (Flanegan et al., 1977; Pettersson et al., 1977). The identity of the spot labelled pUp in Figure 15B was confirmed after elution of the material from the paper; electrophoresis
Fig. 14

Two-Dimensional Analysis by Electrophoresis and Thin-Layer Chromatography of the 5' Terminal RNAase T1-Resistant Nonanucleotide of Poliovirus Virion RNA

$^{32}$P-labelled 5' terminal nonanucleotide was purified as described by Flanegan et al. (1977) and incubated for 15 minutes at 37°C in a HeLa cytoplasmic extract. A control sample was incubated in RSB. Samples were immediately phenol-extracted as described in "Methods and Materials". The phenol phase of the control sample and the aqueous phase of the extract treated sample were precipitated at -20°C with 10 vol of 95% ethanol in the presence of 0.4 M NaCl and 20 μg yeast-carrier tRNA. The precipitates were recovered by centrifugation and redissolved in H$_2$O, and a portion of each was analyzed by two-dimensional electrophoresis (dimension 1) and homochromatography (dimension 2). The remaining portion of each sample was analyzed by ribonuclease digestion as described in Figure 15 and Table 2. The sequences indicated are consistent with the data in Table 2 and with the known structure of the 5' end of poliovirus RNAs (Flanegan, et al., 1977; Pettersson, et al., 1977).
A

VPg-pUUAAACAG

B

pUUAAACAG
FIGURE 15

Ionophoretic Separation at pH 3.5 of Digestion Products Derived From the 5' Terminal Nonanucleotide of Poliovirus RNA

A portion of the $^{32}$P-labelled 5' terminal nonanucleotide samples analyzed in Figure 14 was digested with a mixture of ribonucleases T1, T2, and A, and the products were separated by pH 3.5 ionophoresis on 3 MM paper. The paper was dried and autoradiographed using Kodak XR5 film. (A) digest of untreated nonanucleotide (see Figure 14A). (B) digest of HeLa extract-treated nonanucleotide (see Figure 14B). The positions of XC dye, mononucleotides Cp, Ap, Up and free phosphate, Pi, were determined in a parallel lane. Samples were applied at origin and cathode was below. The positions of pUp and VPg-pUp are indicated.
<table>
<thead>
<tr>
<th>Treatment of the Nonanucleotide</th>
<th>Relative Yield of Products</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VPg-pUp</td>
</tr>
<tr>
<td>None (Fig. 3A)</td>
<td>0.8</td>
</tr>
<tr>
<td>HeLa Extract (Fig. 3B)</td>
<td>0</td>
</tr>
</tbody>
</table>

A portion of the $^{32}$P-labelled nonanucleotide samples analyzed in Figure 14 was digested with a mixture of ribonucleases T1, T2 and A, and the products were separated by pH 3.5-3.5 M paper ionophoresis (Fig. 15). The yield of each product is expressed as the ratio of radioactivity in that product to radioactivity in the Cp residue.
Either the $^{32}$P-labelled 5' terminal nonanucleotide of poliovirus RNA or $^3$H-tyrosine-labelled poliovirus 35S RNA was incubated at 37°C for the indicated times in a sample of HeLa cytoplasmic extract from approximately $1.8 \times 10^5$ cells (A), or HeLa nuclear wash equivalent to approximately $2.5 \times 10^5$ cells (B). VPg removal from RNA was assayed for each time point by phenol extraction of the nonanucleotide or Biogel column chromatography of the $^3$H-tyrosine RNA as described in "Methods and Materials".
Assay time,

$C\;E\;O\;E\;C\;L\;I\;O$

50
100

Assay time, minutes

A HeLa Cytoplasm

$\bullet \bullet$ % $^3\text{H}$ cpm included in Bio Gel column

B HeLa Nuclear Wash

$\circ \circ$ % $^3\text{H}$ cpm in aqueous phase

$0-100$

$0-100$

$0-50$

$0-50$

$0-100$

$0-100$

$0-50$

$0-50$
3H-tyrosine-labelled 35S poliovirion RNA as a substrate. Samples were taken at various times of incubation and chromatographed on Biogel A1.5 M, and the percentage of total radioactivity unlinked from RNA was calculated. 32P-labelled marker 35S RNA was included in each sample to monitor for ribonuclease. In no case was ribonuclease activity sufficient to account for the appearance of significant 3H radioactivity at the position in the Biogel column of free VPg or smaller. For each time of incubation in the cytoplasmic extract, all 3H radioactivity unlinked from RNA appeared as a peak of small molecular weight material at the position of the peak in Figure 12D. The kinetics of removal of VPg as measured by this assay (Fig. 16A, closed circles) are slower than those measured by the phenol partition assay (Fig. 16A, open circles). This is probably due to protease activity present in the cytoplasmic extract capable of degrading large portions of the VPg attached to the nonanucleotide. Reconstruction experiments (data not shown) have determined that treatment with proteinase K or Pronase renders the 5' terminal nonanucleotide aqueous-soluble. As shown earlier (Fig. 12C), the 3H-tyrosine assay for VPg removal is insensitive to contaminating protease, but measures breakage of the tyrosine-phosphate linkage (Fig. 13). This assay is therefore a more accurate representation of the kinetics of breakage of the VPg-RNA bond. At 15 min of incubation in the cytoplasmic extracts, both assays indicate about 90% removal of VPg. This is consistent with the data presented in Table 2, which show that pUp is the predominant 5' terminal structure of the nonanucleotide after 15 min of incubation in a HeLa cytoplasmic extract.

The kinetics of VPg removal in a HeLa nuclear wash are very similar when assayed with the nonanucleotide phenol extraction assay (Fig. 16B,
open circles) or with the column assay (Fig. 16B, closed circles). Furthermore, at each time point, that $^3H$ radioactivity which was unlinked from RNA was found in a peak at the position of intact VPg (as in Fig. 12E). Apparently the HeLa nuclear wash used in these experiments contains the activity able to unlink VPg from poliovirus RNA with much less contaminating protease than is found in cytoplasmic extracts.

Removal of VPg from poliovirus RNA was found to follow the same kinetics (as measured by both assays described above) in extracts of poliovirus-infected HeLa cells as in uninfected HeLa cells (data not shown).

E. Release of VPg from Polio RNA in Other Cell Extracts

Cell-free extracts of three other cell types were assayed using a protocol identical to that used in the experiment described in Figure 12. Figure 17A shows that $^{35}S$ virion RNA labelled with $^{32}P$ (open circles) and $^3H$-tyrosine (closed circles) eluted at the excluded volume of a Biogel A1.5 M column. $^3H$-tyrosine-labelled $^{35}S$ RNA was incubated in a rabbit reticulocyte lysate, and after 15 min, <60% of the $^3H$ radioactivity was found in small molecular weight material unlinked from the $^{32}P$-labelled $^{35}S$ poliovirus RNA marker (Fig. 17B). The same experiment performed with a mouse L cell cell-free extract (Fig. 17C) and a wheat germ extract (Fig. 17D), had similar results, except that the L cell extract left a large portion of the unlinked $^3H$-tyrosine in material the size of VPg, while the wheat germ extracts appeared to degrade VPg. No ability to separate $^3H$-tyrosine from viral RNA was detected in the soluble proteins released from E. coli by sonification (data not shown).
FIGURE 17

Biogel Column Chromatography of Poliovirus RNA

After Incubation in Eucaryotic Cell-Free Extracts

The experiment is identical to the one described in Figure 12, except that the RNA was incubated in (A) buffer, (B) a reticulocyte lysate, (C) a cell-free extract of mouse cells or (D) a wheat germ cell-free extract. Sources of extracts and the method of their preparation are given in "Methods and Materials".
F. Glycerol Gradient Sedimentation of HeLa Unlinking Activity

A 200 μl sample of the HeLa nuclear wash was sedimented through a 15-30% glycerol gradient as described in "Methods and Materials". Unlinking activity on a \(^3\)H-tyrosine-labelled polio RNA sedimented as a peak at approximately 3S (Fig. 18).

G. Magnesium Requirement for Unlinking Activity

To determine whether divalent cations were required for unlinking activity, samples of a HeLa nuclear wash were assayed in the presence of 1 mM MgCl\(_2\). Addition of 2 mM EDTA completely inhibited unlinking activity, and MgCl\(_2\) in 1 mM excess over EDTA reactivated the activity (Table 3). These results indicate that a divalent cation is necessary for unlinking activity.

H. Heat Stability of HeLa Unlinking Activity

To measure the heat stability of unlinking activity, samples of HeLa nuclear wash were incubated at 50 and 37°C for various times, and immediately thereafter assayed at 30°C for the amount of unlinking activity remaining. The half-life of unlinking activity at 50°C was found to be approximately 2 min, and the half-life at 37°C was found to be about 10 min (Table 4).

I. Purification of HeLa Unlinking Activity

To begin purification of the HeLa cell activity which unlinks VPg from poliovirus RNA, a nuclear wash S-100 and cytoplasmic S-100 were prepared as described in "Methods and Materials". These extracts were chromatographed on DEAE-Sephadex A-25 and fractions were assayed for ability to unlink \(^3\)H-tyrosine VPg from polio RNA. Release of VPg was measured by phenol extraction. Both the nuclear S-100 (Fig. 19A) and the cytoplasmic S-100 (Fig. 19B) yield a peak of unlinking activity.
A 200μl sample of HeLa nuclear salt wash was sedimented through a 15-30% glycerol gradient as described in "Methods and Materials". Unlinking activity was assayed by phenol extraction of \( ^{3}H\)-tyrosine-labelled 35S poliovirion RNA. BSA (4.4\S) and cytochrome-c (1.7\S) markers were sedimented through a parallel gradient.
In 100 ies a.

$4.4 \text{ S}$

$1.7 \text{ S}$

$\text{cpm removed from aqueous phase}$

$\text{fraction number}$
**Table 3**

**Divalent Cation Requirement of HeLa Cell Nuclear Wash Activity That Unlinks VPg From Poliovirus 35S RNA**

<table>
<thead>
<tr>
<th>Additions</th>
<th>% $^3$H Radioactivity Phenol-Soluble</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>10</td>
</tr>
<tr>
<td>Nuclear Wash, 1 mM MgCl$_2$</td>
<td>79</td>
</tr>
<tr>
<td>Nuclear Wash, 1 mM MgCl$_2$, 2 mM EDTA</td>
<td>13</td>
</tr>
<tr>
<td>Nuclear Wash, 3 mM MgCl$_2$, 2 mM EDTA</td>
<td>96</td>
</tr>
</tbody>
</table>

Samples of a HeLa nuclear wash were assayed for ability to remove $^3$H-tyrosine-labelled VPg from poliovirus 35S RNA under the indicated reaction conditions. Removal of VPg was measured by the phenol extraction method as described in "Methods and Materials".
### Table 4

**Heat Sensitivity of the HeLa Cell Nuclear Wash Activity That Unlinks VPg From Poliovirus 35S RNA**

<table>
<thead>
<tr>
<th>Temperature and Time of Heating</th>
<th>% Unlinking Activity Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>50°C</strong></td>
<td></td>
</tr>
<tr>
<td>0 min</td>
<td>100</td>
</tr>
<tr>
<td>1 min</td>
<td>75</td>
</tr>
<tr>
<td>2 min</td>
<td>40</td>
</tr>
<tr>
<td>5 min</td>
<td>25</td>
</tr>
<tr>
<td>10 min</td>
<td>15</td>
</tr>
<tr>
<td><strong>37°C</strong></td>
<td></td>
</tr>
<tr>
<td>2 min</td>
<td>80</td>
</tr>
<tr>
<td>6 min</td>
<td>63</td>
</tr>
<tr>
<td>10 min</td>
<td>51</td>
</tr>
</tbody>
</table>

Samples of a HeLa nuclear wash were heated at the indicated temperatures for various times, and immediately thereafter incubated in the presence of \(^{35}\text{S}\)-tyrosine-labelled poliovirus 35S RNA and 1 mM MgCl\(_2\) for 10 min at 30°C. Removal of VPg was measured as in Table 3.
eluting at approximately 120 mM KCl. In both cases, activity also appears with the flow-through for the DEAE-Sephadex column. This activity was not characterized or purified further due to the large amount of protein and contaminating ribonuclease and phosphodiesterase activities in the flow-through (data not shown).

The pool of unlinking enzyme eluting at 120 mM KCl was then chromatographed on cibacron blue sepharose as described in "Methods and Materials". Both the nuclear (Fig. 20A) and cytoplasmic (Fig. 20B) activities elute from the cibacron column at approximately 400-500 mM KCl. The bulk of the proteins applied to this column flow through at 150 mM KCl. The peak fractions of unlinking activity from the cibacron column were pooled and dialyzed against 10 mM Tris pH 7.5, 50 mM KCl, 5 mM β-mercaptoethanol, 1.5 mM MgCl₂, 50% glycerol. This dialysis served to lower the salt concentration and concentrate the sample about 2-3 fold.

J. Purification Table for HeLa Unlinking Enzyme

To quantitate the yield and relative purity of unlinking enzyme at each stage of purification, assays were performed with several dilutions of each enzyme fraction. Figure 21 shows an example of such an enzyme dilution curve for the nuclear cibacron pool. A unit of unlinking enzyme is defined as that amount of enzyme which frees 0.04 pmoles of VPg from polio RNA at 30°C in 30 min under standard incubation conditions. From the linear portion of the plot in Figure 23, the amount of unlinking enzyme in the nuclear cibacron pool can be estimated at approximately 1 unit/2 ml or 500 units/ml. By this procedure enzyme activity was quantitated and protein concentration was measured by the Lowry method. These data are summarized in Table 5. In both the
FIGURE 19

DEAE-Sephadex Chromatography of HeLa Unlinking Enzyme

Nuclear and cytoplasmic S-100 fractions were prepared as described in "Methods and Materials". DEAE-sephadex columns were prepared and equilibrated as described in "Methods and Materials". Samples were applied in 50 mM KCl, TMMG buffer, and % transmission at 280 nm was monitored during elution. Columns were eluted with a 50-200 mM KCl concentration gradient. Salt concentration (-----) was measured by conductance. 5 ml fractions were collected and samples of alternate fractions were assayed for removal of $^3$H VPg from $^3$H-tyrosine-labelled polio RNA (●-●). VPg removal was measured by phenol extraction. A, cytoplasmic S-100. B, nuclear S-100.
Cibacron Blue Sepharose Chromatography of HeLa Unlinking Activity

The pooled fractions from the peak of unlinking activity that eluted from DEAE-sephadex at 120 mM KCl (Figure 19) were applied to Cibacron-sepharose columns, and the columns were eluted and fractionated as described in "Methods and Materials". Salt concentration (-----), % transmission at 280 nm (-----), and unlinking activity (○-○), were measured as for Figure 19. A, cytoplasmic DEAE-sephadex pool of unlinking activity. B, nuclear DEAE-sephadex pool of unlinking activity.
FIGURE 21
Dilution of Nuclear Cibacron Pool of Unlinking Enzyme

Standard unlinking activity assay reactions were performed with 0.1 pmoles $^3$H-tyrosine-labelled poliovirus RNA as substrate but with different amounts of nuclear cibacron pool unlinking enzyme. Removal of VPg from RNA was measured by phenol extraction after the 30 minute incubation at 30°C.
<table>
<thead>
<tr>
<th>Enzyme Fraction</th>
<th>mg Protein</th>
<th>Units of Enzyme</th>
<th>Yield of Enzyme</th>
<th>Specific Activity</th>
<th>Net Purifications</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cytoplasmic S-100</td>
<td>500</td>
<td>4,800</td>
<td>100%</td>
<td>9.5</td>
<td>1</td>
</tr>
<tr>
<td>Cytoplasmic DEAE-Sephadex</td>
<td>18</td>
<td>2,200</td>
<td>45%</td>
<td>120</td>
<td>13</td>
</tr>
<tr>
<td>Cytoplasmic Cibacron Pool</td>
<td>0.32</td>
<td>760</td>
<td>16%</td>
<td>2,400</td>
<td>250</td>
</tr>
<tr>
<td>Nuclear S-100</td>
<td>84</td>
<td>12,000</td>
<td>100%</td>
<td>140</td>
<td>1</td>
</tr>
<tr>
<td>Nuclear DEAE-Sephadex Pool</td>
<td>1.6</td>
<td>5,400</td>
<td>45%</td>
<td>3,300</td>
<td>24</td>
</tr>
<tr>
<td>Nuclear Cibacron Pool</td>
<td>0.07</td>
<td>3,000</td>
<td>25%</td>
<td>42,000</td>
<td>280</td>
</tr>
</tbody>
</table>

a) 1 unit = amount of enzyme which will remove 0.04 pmoles of VPg from poliovirus RNA in 30 min at 30°C at substrate concentration of 0.1 pmole polio RNA/20 μl reaction

b) based on relative yield of enzyme units with respect to S-100 starting fraction.

c) units unlinking enzyme/mg protein

d) based on specific activity of each fraction relative to S-100 starting fraction
nuclear and cytoplasmic cibacron pools, approximately 15-25% yield of unlinking enzyme, and about 250-fold purification is achieved relative to the respective S-100 starting fractions. The nuclear fraction yields more enzyme of higher specific activity than does the cytoplasmic fraction.

K. SDS Acrylamide Gel Electrophoresis of HeLa Unlinking Enzyme

A portion of the nuclear cibacron pool was concentrated by binding to a small DEAE-Sephadex column followed by elution with 150 mM KCl in TMMG buffer. 20 μl of the concentrated sample (approximately 2 μg protein), and a 10 μl sample of the nuclear S-100 fraction were diluted 1:1 with 2X Laemmli sample buffer and electrophoreses through a 12% acrylamide gel (Laemmli, 1970) with a mixture of molecular weight markers. The gel was stained with comassie brilliant blue and photographed (Fig. 22). The nuclear cibacron pool of unlinking activity (Fig. 22, Lane 3) is enriched with respect to the nuclear S-100 starting material (Fig. 22, Lane 3) for a 27,000 molecular weight polypeptide. A protein of this identical molecular weight is enriched in two completely independent preparations of cibacron purified nuclear unlinking enzyme, as well as the cibacron fraction of unlinking activity obtained from the cytoplasm (data not shown). The cytoplasmic cibacron pool, however, contains a larger quantity of other polypeptides of various sizes than does the nuclear cibacron pool.

L. Substrate Curve for Removal of VPg from 35S Poliovirus RNA

To determine the optimum polio RNA concentration for removal of VPg by HeLa nuclear cibacron fraction of unlinking enzyme, assays were performed under identical conditions but with various concentrations of polio RNA substrate. The amount of VPg freed (as measured by phenol
FIGURE 22

SDS Acrylamide Gel Electrophoresis of HeLa Unlinking Enzyme Fractions

Cell fractionation and purification of unlinking activity were performed as described in "Methods and Materials". Samples were prepared and electrophoresed as described in text of "Results" section. Lanes 1 and 4, molecular weight markers Phosphorylase b (94,000), Bovine Serum albumen (67,000), Ovalbumen (43,000), Carbonic anhydrase (30,000), Soybean trypsin inhibitor (20,100), and α-Lactalbumen (14,400). Lane 2, approximately 45 µg nuclear S-100. Lane 3, approximately 2 µg nuclear cibacron pool of unlinking enzyme. Gel was stained with comassie brilliant blue.
extraction) by 1 unit of enzyme in 30 min at 30°C is proportional to polio RNA substrate concentration up to about 10 nM, or 0.2 pmole (0.5 μg) 35S RNA/20 μl reaction volume, (Fig. 23).

K. Gel Filtration Assay of Pooled Cibacron Unlinking Enzyme

To confirm that the activity obtained by cibacron blue chromatography represents an enzyme able to remove VPG from polio RNA, the Biogel A1.5 M column assay was employed. Three samples containing 0.2 pmole (0.5 μg) 3H-tyrosine-labelled polio RNA plus 1200 cpm 32P polio RNA were incubated under standard assay conditions at 30°C for 1 h either with or without cibacron purified enzyme. The samples were then processed and chromatographed or Biogel A1.5 M in the presence of SDS as described in "Methods and Materials". The sample receiving no enzyme contained virtually all of the 3H radioactivity eluting with the excluded volume, coincident with the 32P 35S RNA marker profile (Fig. 24A). This is consistent with the covalent attachment of VPG to RNA. The samples which were incubated with either 4 μl of nuclear cibacron pool (Fig. 24B) or 4 μl of the cytoplasmic cibacron pool (Fig. 24C) contained a large portion of 3H radioactivity eluting like free VPG, which runs at the position of cytochrome-c. In neither case was the 32P 35S polio RNA marker significantly degraded. Therefore, these cibacron pools contain activity able to release VPG from poliovirus RNA. The protease activity detected in the crude cytoplasmic extract (Fig. 12D) is apparently absent from the cytoplasmic cibacron pool.

N. Agarose Gel Electrophoresis of Unlinking Enzyme-Treated Poliovirus RNA

To examine the degree of intactness of poliovirus RNA after incubation with cibacron sepharose purified unlinking enzyme, agarose gel electrophoresis was employed. 0.5 μg (0.2 pmoles) samples of poliovirus RNA
Incubations were performed under standard conditions for 30 minutes at 30°C. 20μl reactions contained 1 unit of nuclear cibacron pool unlinking enzyme and various concentrations of $^3$H-tyrosine labelled poliovirus RNA. Removal of VPg from RNA was measured by phenol extraction as described in "Methods and Materials". 35S Poliovirus RNA concentration is expressed in terms of molarity of 5' ends.
pmoles VPg released from RNA/30 min

pmoles 35S polio RNA/20μl
FIGURE 24

Gel Filtration Assay of
Cibacron-Sepharose Purified Unlinking Enzyme

20 μl reactions containing 0.2 pmoles \(^{3}H\)-tyrosine-labelled poliovirion RNA and 1200 cpm \(^{32}P\) poliovirion RNA were incubated with 4 μl of cibacron sepharose purified unlinking activity for 1 h at 30°C or with no enzyme under identical conditions. Samples were chromatographed through Biogel A1.5M as described in "Methods and Materials". \(^{3}H\) radioactivity in VPg (○○○) and \(^{32}P\) radioactivity in polio RNA (●●●) were measured by liquid scintillation counting. The column was calibrated with blue dextran 2000 (BD), cytochrome-c (Cy), and bromphenol blue (BPB) in a parallel run. A, control sample, with no enzyme treatment. B, sample treated with nuclear cibacron pool. C, sample treated with cytoplasmic pool.
were incubated with either 4\textmu l buffer, 4\textmu l nuclear cibacron fraction or with 4\textmu l cytoplasmic cibacron fraction for 1 h. These conditions are sufficient to remove approximately 60-80\% of VPg by Biogel column assay (see Fig. 24). At the end of the incubation period, each sample was heated to 70^\circ C for 1 min to break up RNA aggregates, and electrophoresed through a 1\% agarose tube gel. The ethidium bromide stained gels were photographed. In all three samples full length polio RNA is evident, although the control sample (Fig 25A) contained perhaps twice as much full length RNA as either the sample treated with nuclear cibacron pool (Fig. 25B) or with the cytoplasmic cibacron pool (Fig. 25C). Both enzyme preparations appear to reduce the amount of full length polio RNA to comparable degrees under these conditions.

0. Infectivity of Poliovirus RNA Treated with HeLa Unlinking Enzyme

Data presented above indicated that incubation of polio RNA with cibacron sepharose purified unlinking activity left a significant portion of the RNA intact as judged by electrophoretic mobility in 1\% agarose (Fig. 25). To determine whether or not polio RNA treated with HeLa unlinking activity remain biologically active, RNA infectivity assays were performed. In interpreting this experiment, the assumption is made that only full length polio RNA molecules can be infectious. Therefore, RNA samples were analyzed for amount of 35S RNA immediately prior to being assayed for infectivity.

A mixture of 0.16 pmole (0.4\mu g) \textsuperscript{3}H-tyrosine-labelled 35S polio RNA and 400 cpm of \textsuperscript{32}P 35S polio RNA (final specific activity approximately 1000 cpm \textsuperscript{32}P/\mu g RNA) was incubated with 6 units of nuclear cibacron unlinking enzyme at 30^\circ C for 1 h. A control sample contained no enzyme. After incubation, half of each sample was electrophoresed
FIGURE 25

Agarose Gel Electrophoresis of
Unlinking Enzyme Treated Poliovirus RNA

0.5 Mg samples of poliovirus virion RNA were electrophoresed through 1% agarose as described in "Methods and Materials" after treatment for 1 h at 30°C with (A) buffer, (B) 4 μl nuclear cibacron purified unlinking enzyme, or (C) 4 μl cytoplasmic cibacron purified unlinking enzyme.
through 1% agarose as described for Figure 25, and stained with ethidium bromide. The band of full length polio RNA was sliced out of the gel, the agarose melted by brief boiling in 0.5 ml of 5% acetic acid, and \(^{3}H\) and \(^{32}P\) radioactivity were measured. The amount of \(^{32}P\) radioactivity in the \(35S\) RNA band provides an estimate of the number of micrograms of full length RNA remaining in the sample after the incubation time; \\
\[(\text{mgRNA} = \text{specific activity} \times {^{32}P\text{cpm}})\]. The amount of \(^{3}H\) radioactivity in the \(35S\) RNA band provides a measure of the number of VPg molecules present. The ratio of \(^{3}H\) radioactivity to \(^{32}P\) radioactivity provides a relative measure of the number of VPg molecules per \(35S\) RNA molecule.

The control sample is assumed to contain one VPg molecule per \(35S\) RNA molecule, for with no added enzyme, all VPg remains attached to RNA. The number of VPg molecules per \(35S\) RNA molecule in the incubated sample was calculated by comparing the \(^{3}H/^{32}P\) ratio for that sample to the \(^{3}H/^{32}P\) ratio for the control sample. The other half of each sample was assayed for infectious polio RNA as described in "Methods and Materials".

These data are presented in Table 6. RNA infectivity is expressed as pfu/\(\mu\)g full length polio RNA. The sample which was incubated with enzyme had a \(^{3}H/^{32}P\) ratio in the \(35S\) RNA band which was 20% of that of the control. Therefore, that sample contained approximately 0.2 covalently attached VPg molecules per \(35S\) RNA molecule (or VPg was removed from 80% of the RNA molecules). Assuming that only full length molecules contribute to infectivity of these samples, then the RNA incubated with unlinking enzyme was 60% as infectious as the sample with all VPg intact. Under the conditions used in this experiment, infectivity is proportional to amount of added RNA (Spector and Baltimore, 1974). Therefore, if only \(35S\) RNA with intact VPg were infectious, then the unlinking enzyme
### TABLE 6

**INFECTIVITY OF 35S POLIOVIRUS RNA TREATED WITH HELA UNLINKING ENZYME**

<table>
<thead>
<tr>
<th>RNA Sample</th>
<th>cpm (^{32}\text{P}) in 35S RNA</th>
<th>Mg 35S RNA</th>
<th>cpm (^{3}\text{H}) in 35S RNA</th>
<th>(\frac{\text{cpm} \text{ }^{3}\text{H} \text{ cpm} \text{ }^{32}\text{P}}{1000 \text{ cpm} \text{ }^{32}\text{P}})</th>
<th>VPg Total pfu</th>
<th>(\frac{\text{pfu} \text{ Mg 35S RNA}}{\text{Mg 35S RNA}})</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control RNA (not treated with enzyme)</td>
<td>334</td>
<td>0.33</td>
<td>380</td>
<td>1.14</td>
<td>1.0</td>
<td>2200</td>
</tr>
<tr>
<td>RNA treated with unlinking enzyme</td>
<td>213</td>
<td>0.21</td>
<td>50</td>
<td>0.23</td>
<td>0.2</td>
<td>860</td>
</tr>
</tbody>
</table>

a) Half of each sample was electrophoresed through 1% agarose; the gel was stained with ethidium bromide; the 35S RNA band was cut out, and \(^{3}\text{H}\) and \(^{32}\text{P}\) radioactivity was measured.

b) calculated from \(\text{Mg 35S RNA} = \frac{(\text{cpm }^{32}\text{P} \text{ in 35S RNA})}{1000 \text{ cpm }^{32}\text{P}}\) \(\text{Mg RNA}\)

c) (VPg/35S RNA molecule) is assumed to be equal to one for control RNA; for enzyme treated RNA, (VPg/35S RNA molecule) = 0.23. (see text for further explanation).

d) The other half of each sample was assayed for infectivity by plaque assay:

![Graph showing infectivity of samples](image)
treated sample would be expected to contain approximately 20% of the infectivity of the control sample. The finding of 60% of control infectivity fails to support the idea that 5' terminal VPg is necessary for infection by poliovirus RNA.

4) DISCUSSION

The finding that VPg is removed from poliovirus RNA by an activity in extracts of HeLa cells supports the hypothesis that the maturation of poliovirus messenger RNA during poliovirus replication involves the removal of VPg from the 5' end of intracellular RNA molecules. The fact that the activity is able to remove VPg from exogenously added poliovirus RNA and leave the same 5' end structure (pUUAAACAG) as found on messenger RNA further supports the model that VPg is unlinked from poliovirus RNA by the specific cleavage of the bond between the tyrosine residue in VPg and the 5' terminal phosphate of poliovirus RNA (Flanagan et al., 1977; Pettersson et al., 1977). Further evidence for the specificity of this unlinking activity is the fact that a preparation from HeLa cell nuclei cleaves VPg from the RNA, leaving VPg intact with the tyrosine of VPg completely free of covalently bound RNA.

This chapter describes three assays for activity able to unlink VPg from poliovirus RNA. One involves phenol extraction of the 5' terminal nonanucleotide labelled with $^{32}\text{P}$, and the second utilizes Biogel column chromatography of $^{35}\text{S}$ RNA with $^{3}\text{H}$-tyrosine label in VPg. The latter assay is not prone to artifactual results due to contaminating proteases, and in the absence of appreciable nuclease, measures only breakage of the tyrosine-phosphate linkage between VPg and the RNA. The 5' terminal nonanucleotide assay is sensitive to proteolytic removal of VPg. When assaying preparations of unlinking activity which are relatively free
of protease, we find that the phenol extraction assay is more rapid and convenient to use. A third assay utilizes the same phenol extraction procedure, but the substrate is $^3$H-tyrosine-labelled 35S RNA. Unlinking activity is detected by the removal of $^3$H radioactivity from the aqueous phase. This assay is rapid but can be used only under conditions where ribonuclease activity does not interfere.

A partially purified preparation of unlinking activity can be obtained by a 0.2 M KCl wash of uninfected HeLa cell nuclei. Our finding that this activity sediments as a 3S peak on a glycerol gradient, is heat-labile and is found in uninfected cells is consistent with a single cellular enzyme being responsible. Furthermore, both the cytoplasmic unlinking activity and the nuclear wash activity co-chromatograph on DEAE-Sephadex and cibacron blue sepharose chromatography. These activities probably represent the same enzyme. A 27,000 molecular weight protein is enriched in the cibacron fraction of both. The fact that this enzyme is found in the cytoplasm of both infected and uninfected cells is consistent with its possible role in polio replication, which occurs in the cytoplasm.

Although a significant portion of cellular unlinking activity is recovered from the nuclear wash, there is no evidence that the enzyme is involved in normal nuclear processes. The cell fractionation procedure used in these experiments is relatively crude, and there is undoubtedly significant cross-contamination between the nuclear and cytoplasmic preparations. The normal function of this enzyme in uninfected cells is not known, and there is no evidence that the unlinking activities measured in extracts of rabbit reticulocytes, L cells or wheat germ are due to an enzyme functionally related to the one in HeLa cells.
Chapter V describes experiments designed to learn more about the substrate specificity of HeLa unlinking enzyme, and about its role in uninfected cells.

Polio RNA treated with unlinking activity is 60% as infectious as untreated RNA when corrections are made for partial degradation of the treated sample. This remaining infectivity could be entirely due to the approximately 20% of all molecules which retained VPg after incubation. A more likely possibility is that poliovirus RNA molecules, with or without 5' terminal VPg have equal intrinsic infectivities, and the 40% lower calculated infectivity for unlinking enzyme treated $^{35}$S RNA (Table 6) is due to inherent variability in the RNA infectivity assay, or an overestimation of the amount of full length molecules in the $^{35}$S RNA gel sample. This interpretation is consistent with the observation that 5' terminal protein of poliovirus can be proteolytically degraded, and the RNA of poliovirus retains normal infectivity (Nomoto et al., 1977).

Isolated polio polysomal RNA, which has pUp at its 5' end, is also approximately as infectious as virion RNA (Ambros, Hewlett, Baltimore, unpublished results). Preparations of polysomal RNA, however, contain a variable but significant portion of polio RNA with 5' terminal VPg (Pettersson and Ambros, unpublished observation). Therefore, the infectivity experiment described in this chapter provides additional evidence that 5' terminal VPg is not necessary for the initiation of infection by polio RNA. Furthermore, these infectivity results, considered together with the structural data presented in Figures 13, 14 and 15, indicate that polysomal RNA prepared from infected cells, and virion RNA which has had VPg removed in vitro by purified HeLa unlinking enzyme, are structurally and biologically equivalent. This further
supports the model that this enzyme could generate polio mRNA \textit{in vivo}
by the cleavage of VPg from polio RNA.
CHAPTER V
PRELIMINARY STUDY OF SUBSTRATE SPECIFICITY OF
HELA UNLINKING ENZYME

1) INTRODUCTION

Since the enzyme which is able to cleave the tyrosine-phosphate linkage between VPg and the 5' end of poliovirus RNA (unlinking enzyme) is a normal cellular enzyme, it seems reasonable to postulate that protein-nucleic acid linkages via phosphodiester bonds to tyrosine may be found in normal cells, and that poliovirus uses for maturation of poliovirus messenger RNA a cellular enzyme normally involved in the cleavage of such linkages. One such linkage is known in bacteria--the joining of a uridylate residue to the regulatory protein (PII) of glutamine synthetase (Adler, Purich and Stadtman, 1975) --but no equivalent bond in mammalian cells has been described.

In order to find the normal substrate for HeLa unlinking enzyme in uninfected cells, it is first necessary to learn more about its substrate specificity. A preliminary study is described in this chapter. Derivatives of poliovirus RNA-VPg complex were made by protease and ribonuclease digestion, and those derivatives were tested for cleavage by HeLa unlinking enzyme. Intact VPg is not necessary for cleavage of the tyrosine-RNA linkage, but a nucleic acid substituent longer than pUp is essential for cleavage of the linkage.

An experiment designed to detect covalent linkage of tyrosine to nucleic acid in normal HeLa cells is described.

2) METHODS AND MATERIALS

A. General Methods
Preparation of poliovirus 35S 3H-tyrosine-labelled RNA, enzymatic digestions, 3 MM paper ionophoresis and Biogel column chromatography were as described in the "Methods and Materials" sections of previous chapters.

B. 3H-tyrosine-Labelling of Aqueous Soluble HeLa Nucleic Acid

2 x 10^8 HeLa cells (4 x 10^5/ml) were labelled with 4 mCi 3H-tyrosine in 500 ml MEM (lacking unlabelled tyrosine) for 4 h at 37°C. During this time incorporation of 3H radioactivity into TCA precipitable material continued to increase linearly until 3.5-4 h. Total incorporation was 3 x 10^8 cpm 3H after 4 h of labelling. Cells were harvested and lysed with 4 ml 0.5% SDS, 20 mM Tris pH 7.5, 1 mM EDTA. To half of the lysed cells, 20 mg protease-K was added and that sample was digested at 37°C for 30 min. 50 ml of 20 mM Tris pH 7.5, 1 mM EDTA, 150 mM NaCl was added to both samples, and they were extracted twice with phenol/chloroform and the aqueous phases were precipitated with ethanol. The nucleic acid was recovered by centrifugation and lyophilized. The protease-K-treated and untreated samples were then each redissolved in 5 ml of 10 mM Tris pH 7.5, 0.5 mM EDTA. 6.44 gm CsCl and 1.2 gm guanidine HCl were added to each sample and dissolved. A 5.5 ml portion of each was transferred to an SW 50.1 cellulose nitrate tube, and the samples were centrifuged in an SW 50.1 rotor at 38K for 60 h at 18°C. Fractions were collected through a hole in the bottom of each tube (just above the solid salt pellet). Samples of each fraction were assayed for OD_{260} and TCA precipitable radioactivity. A sample of each fraction was also diluted 10 fold and assayed for aqueous soluble TCA precipitable radioactivity after phenol extraction.
3) **RESULTS**

A. **Treatment of Protease-K Digested Polio RNA with HeLa Unlinking Enzyme**

$^3$H-tyrosine-labelled $^{35}$S polio RNA was treated with protease K to digest away all of VPg but "K-peptide" which remains linked to the RNA via $^3$H-tyrosine (see Figure 12C). This material was phenol extracted, ethanol precipitated, and used as a substrate in the standard unlinking enzyme assay. $^{32}$P-labelled $^{35}$S polio RNA was included in the incubation. Removal of K-peptide was measured by Biogel column chromatography. $^3$H-tyrosine-labelled protease-K treated RNA and $^{32}$P RNA co-elute at the void volume fractions of the column (Fig. 26A). After incubation with 2.5 units nuclear cibacron unlinking enzyme, the $^{32}$P RNA is relatively intact, but small molecular weight $^3$H-labelled material is released (Fig. 26B). This $^3$H-labelled material runs near the included volume of the column, consistent with the expected small size of the protease-K-resistant K-peptide. Full size VPg is apparently not required for cleavage of the 5' terminal tyrosine-phosphate bond of polio $^{35}$S RNA.

B. **Treatment of Ribonuclease Digested Poliovirus RNA with HeLa Unlinking Enzyme**

As shown in Chapter IV, crude extracts of HeLa cells are able to remove VPg from the 5' terminal T1 resistant oligonucleotide of poliovirus. To confirm that purified HeLa unlinking enzyme retains activity on this substrate, $^3$H-tyrosine-labelled 5' terminal oligonucleotide was generated by T1 digestion of $^{35}$S poliovirus RNA. This material was incubated with 2.5 units of unlinking enzyme (5 µl of nuclear cibacron fraction) in 15 µl reaction volume. A control sample of 5' terminal $^3$H-tyrosine-labelled T1 oligonucleotide was incubated with no added unlinking enzyme. Both samples were analyzed by 3 MM paper ionophoresis...
FIGURE 26

Gel Filtration Assay for Removal of K-peptide from Poliovirus 35S RNA by Cibacron Purified Unlinking Enzyme

Reaction conditions and chromatographic procedures were exactly as described in caption to Figure 24. Protease-K treated poliovirus RNA was prepared as described in the text, and chromatographed on Biogel A1.5 before (A) and after (B) treatment with nuclear cibacron pool unlinking enzyme.
after protease-K digestion. The sample which was not incubated with unlinking activity contained $^3$H radioactivity which ran near the position of the XC dye marker (Fig. 27A). This material represents the $^3$H-tyrosine containing K-peptide linked to a T1-resistant oligonucleotide. The incubated sample contained $^3$H radioactivity in free K-peptide, and some radioactivity in K-peptide attached to the oligonucleotide (Fig. 27B). Under these conditions, the purified unlinking enzyme was able to cleave a portion of the protein-nucleic acid linkage between VPg and the 5' terminal T1 oligonucleotide of poliovirus RNA. This is consistent with the activity measured in crude cytoplasmic and nuclear fractions (Chapter IV) being the same enzyme found in this purified fraction.

Similar experiments were performed to test whether or not VPg-pUp or VPg-pU can be cleaved by the nuclear cibacron unlinking enzyme fraction. 0.4 pmole (1 μg) of $^3$H-tyrosine-labelled 35S poliovirus RNA in 10 μl TMHG were added to each of 5 vials. One vial (control) received no addition of RNase. In two other vials, $^3$H-tyrosine-labelled VPg-pUp was generated by the addition of 1 μl of a 1/10 dilution of the standard ribonucleases T1, T2, and A mixture. In the remaining two vials, $^3$H-tyrosine-labelled VPg-pU was generated by the addition of 1 μl containing 0.2 μg P1 nuclease. All five vials were incubated at 37°C for 1 h. One tube each of VPg-pUp and VPg-pU was then further incubated with no addition, while the other tube of each derivative received 2.5 units nuclear cibacron fraction of unlinking enzyme. The vial containing intact 35S RNA (no RNase digestion) also received 2.5 units of unlinking enzyme. All five tubes were incubated at 30°C for 18 h. All samples were then digested with 1 μl of 10 mg/ml protease-K for 1 h at 37°C. This treatment would be expected to generate KpUp and KpU in the case of the
FIGURE 27

pH 3.5 3 MM Paper Ionophoresis of Enzymatic Digestion Products of

$^3$H-Tyrosine-labelled Poliovirus RNA

$^3$H-tyrosine-labelled VPg attached to the 5' terminal T1 oligonucleotide of poliovirus RNA (VPg-oligo), VPg-pUp, and VPg-pU were generated by ribonuclease digestion of poliovirus RNA as described in the text. Samples were then treated with either buffer or cibacron pool unlinking enzyme for 18 hours at 30 C. All samples were then treated with protease-K and subjected to pH 3.5 3 MM paper ionophoresis as described for Figure 7. A, VPg-oligo control, treated with protease-K. B, VPg-oligo treated with unlinking enzyme followed by protease-K. C, $^{35}$S poliovirus RNA treated first with unlinking enzyme, followed by ribonucleases T1, T2, and A, and finally protease-K. D, VPg-pUp control sample treated with protease-K. E, VPg-pUp treated with unlinking enzyme, followed by protease-K. F, VPg-pU control sample treated with protease-K. G, VPg-pU treated with unlinking activity followed by protease-K. 0, origin. XC, xylene cyanol dye marker. Cathode was on left.
control sample. If, during the 18 h incubation with unlinking enzyme, the bond between VPg and either pUp or pU were broken, free K-peptide would be generated following protease-K digestion. All samples were analyzed by 3 MM paper ionophoresis exactly as in Figure 7. Unlinking activity would be expected to remove VPg from 35S RNA as usual. Figure 27C confirms this, showing that 3H-tyrosine-labelled free K-peptide is generated by unlinking enzyme followed by protease-K digestion. 3H radioactivity in both the control VPg-pUp sample (Fig. 27D), and unlinking enzyme treated VPg-pUp sample (Fig. 27E), migrates as KpUp after protease-K treatment. No RNA-free K-peptide is evident. Similarly, 3H radioactivity in both the control VPg-pU sample (Fig. 27F), and incubated VPg-pU sample (Fig. 27G), migrates as K-pU after protease-K digestion. These data demonstrate that VPg-pUp and VPg-pU are resistant to unlinking activity under conditions where VPg is removed from 35S polio RNA and from the 5' terminal T1 oligonucleotide of polio RNA. The apparent incomplete digestion of the linkage of VPg to the oligonucleotide under conditions where the linkage to 35S RNA was completely broken may imply that the oligonucleotide is a slightly poorer substrate for unlinking enzyme. However, this result may be an artifact caused by the fact that these two incubations, although carried out under otherwise identical conditions, were performed on separate occasions using different samples of enzyme.

C. Treatment of KpUp with Unlinking Enzyme

In the previous experiment, there was a danger that the presence of ribonucleases, or nucleoside-monophosphates, although in low concentrations, might have inhibited the unlinking enzyme, thereby causing a false negative result for cleavage of VPg-pUp or VPg-pU. Therefore, electrophoretically
purified KpUp was tested as a substrate for unlinking enzyme. KpUp was generated by enzymatic digestion of $^3$H-tyrosine-labelled poliovirus RNA as outlined for Figure 7A, and was purified by pH 3.5 3 MM paper ionophoresis, followed by elution from the dried paper with water. The KpUp solution was freeze-dried, and the sample was redissolved in 0.1 mM EDTA pH 7.5 at 0.2 pmoles/μl. An identical piece of the ionophoresis paper was cut from a region adjacent to the area which contained KpUp and processed in parallel to confirm that no inhibitor of unlinking enzyme is eluted from the paper by this procedure. Samples containing 0.4 pmoles KpUp were re-ionophoresed on 3 MM paper at pH 3.5 both before (Fig. 28A), and after (Fig. 28B), treatment with 5 units of unlinking activity under standard conditions for 18 h. No RNA-free K-peptide was released from K-pUp. As a control, unlinking enzyme digestions of $^3$H-tyrosine-labelled 35S RNA were performed in parallel. One incubation used buffer containing residue eluted from 3 MM paper, and the other incubation contained fresh buffer. VPg was removed from the RNA as expected in both reactions (data not shown). Therefore, the lack of cleavage of KpUp by HeLa unlinking enzyme is not an artifact caused by co-elution from 3 MM paper of KpUp and some unknown inhibitor of unlinking activity.

As mentioned in chapters II and III, venom phosphodiesterase cleaves the bond between VPg and either pUp or 1U. The data described above indicate that the cibacron fraction of unlinking enzyme does not contain such a venom phosphodiesterase activity. Earlier preparations of cibacron purified unlinking activity were found to contain some venom-like activity as assayed using 5' para-nitro phenyl-dTMP as substrate. This activity was found only when the starting material for purification
FIGURE 28

pH 3.5 3 MM Paper Ionophoresis of $^3$H-Tyrosine-labelled K-pUp after treatment with Unlinking Enzyme

$^3$H-Tyrosine-labelled K-pUp was generated by enzymatic digestion of poliovirus TNA and purified by pH 3.5 3 MM paper ionophoresis as described in the text. Samples of K-pUp were then ionophoresed at pH 3.5 on 3 MM paper before (A) and after (B) treatment with 5 units of unlinking activity for 18 h at 30°C. Procedures for ionophoresis were exactly as in Figure 27. O, origin. XC, xylene cyanol.
If

Fraction number

- cpn

Fraction number

- cpn

K-pUp

0

X C
was an S-20 supernatant fraction, rather than an S-100 fraction.

D. Labelling of Uninfected HeLa Cells With $^3$H-Tyrosine

2 x $10^8$ HeLa cells were labelled for 4 h with $^3$H-tyrosine as described in "Methods and Materials". Half the cells were lysed with SDS and treated with protease-K followed by phenol extraction. The other half of the cells were lysed and phenol extracted with no protease-K treatment. Each aqueous soluble fraction was precipitated with ethanol and fractionated by CsCl-guanidine HCl equilibrium density gradient centrifugation as described in "Methods and Materials". No aqueous-soluble TCA precipitable $^3$H radioactivity was found in any fraction of either gradient (data not shown).

4) DISCUSSION

The linkage between VPg and either pUp or pU is not cleaved by HeLa unlinking enzyme under conditions where the linkage between VPg and polio 35S RNA or the 5' terminal T1 oligonucleotide of polio RNA is cleaved. The linkage between the protease-K resistant oligopeptide of VPg (K-peptide) and 35S RNA is cleaved by unlinking enzyme, but K-pUp is not. These results indicate that the unlinking enzyme requires a polynucleotide as part of its substrate, but does not require full size VPg. Other necessary structure features for cleavage of a protein-nucleic acid linkage by this enzyme have not been investigated. Other viral RNA molecules which have 5' terminal proteins have not yet been tested as substrates. Adenovirus protein-DNA complex has been treated with HeLa unlinking enzyme, but no satisfactory result, either positive or negative, has been yet obtained (G. Pearson, personal communication).

MMV DNA-protein complex may be a likely natural substrate for
unlinking enzyme, for there is evidence suggesting that the MNV terminal protein might be removed from DNA prior to encapsidation (see Chapter I).

At about 3.5 h after polio infection, several species of viral RNA's, (nascent plus strands, double strands, negative strand of RI), have VPg attached. Unlinking activity is easily detectable in extracts of infected cells at this time, so why is VPg not removed from these RNA's? Polio double strands, RI RNA, and negative strand RNA have not yet been tested for in vitro removal of VPg by unlinking enzyme. VPg linked to the 5' end of duplex RNA may be resistant to removal by unlinking enzyme because of a requirement by the enzyme for single stranded RNA. The linkage between VPg and Poly U of negative strand may be resistant to cleavage because the enzyme might recognize only a specific nucleotide sequence found near the 5' end of positive strands. It is possible that VPg of nascent positive strands binds specifically to some large structure in the infected cell in such a way that under some conditions the protein-nucleic acid linkage is protected from cleavage by unlinking enzyme. This possibility is incorporated into a working hypothesis for future study of the function of VPg and is discussed in Chapter VI.

It was postulated that cellular tyrosine-nucleic acid linkages might be the natural substrate of unlinking enzyme. The one experiment described in this chapter which attempted to detect $^3$H-tyrosine linked to HeLa cell nucleic acid failed to demonstrate any such structures. No $^3$H-tyrosine was found associated with either the RNA or DNA fractions of a CsCl-Guanidine HCl equilibrium density gradient. This nucleic acid was phenol extracted, so the possibility exists that HeLa cell structures containing tyrosine-nucleic acid linkages are not aqueous-soluble under
extraction conditions. However, material that was treated with protease-K, (a treatment which does not break the tyrosine-RNA linkage of poliovirus), prior to phenol extraction was also free of \(^{3}H\)-tyrosine label in this experiment. Another possibility is that tyrosine is covalently attached to HeLa nucleic acid only transiently, or with kinetics of an order much greater than the 4 h labelling period employed in this experiment. Such linkages may exist in such low concentration per cell that they are undetectable among the quantity of labelled material analyzed here. Finally, the substrate specificity of HeLa unlinking enzyme could be quite broad, and whereas the tyrosine-phosphate linkage of poliovirus is an appropriate substrate, that particular linkage may not have a precise analog in uninfected cells.
CHAPTER VI

SUMMARY AND GENERAL DISCUSSION

1) SUMMARY

VPg of poliovirus is a 12000 molecular weight protein covalently bound through a tyrosine residue to the 5'-terminal phosphate of polio RNA. At 3.5 h after infection, the only detectable 5' end structure on any size class of nascent polio molecules is VPg. Both infected and uninfected HeLa cells contain an enzymatic activity which breaks the linkage between the polio 5' terminal phosphate and the tyrosine of VPg. This enzyme has been purified approximately 250 fold from an s-100 supernatant fraction of HeLa cytoplasm and from a salt wash of HeLa nuclei. This enzyme (called unlinking enzyme) will cleave the protease-K resistant oligopeptide from the 5' end of protease-K treated poliovirus RNA, as well as intact VPg from normal RNA. It will not unlink pU or pUp from VPg.

2) THE ROLES OF VPg AND HEILA UNLINKING ENZYME IN POLIOVIRUS REPLICATION

A. Role of VPg in Early Events of Infection

Removal of VPg with HeLa unlinking enzyme or degradation of VPg by protease does not appreciably decrease infectivity of polio 35S RNA. Therefore, at this point there is no evidence that VPg is necessary for initiation of infection.

Because poliovirus mRNA, which lacks VPg, is found on polyribosomes, and each molecule therefore undergoes multiple initiation events, 5' terminal VPg cannot be necessary for initiation of translation. At this point it is unknown whether or not VPg must be removed before a polio molecule can initiate translation; RNA isolated from virions which
contains VPg, can direct synthesis of polio proteins in cell extracts, but these extracts contain activity which rapidly removes VPg from added polio RNA (see Fig. 17).

B. Role of VPg in Polio RNA Synthesis

A role for VPg as a primer for polio RNA synthesis has been mentioned several times in this thesis and elsewhere (Flanegan et al., 1977; Nomoto, et al., 1977). The finding that VPg is attached to nascent polio RNA molecules at 3.5 h after infection is consistent with this hypothesis, but of course does not prove it. It is conceivable that VPg is attached to each growing chain very soon after RNA synthesis has been initiated by some other mechanism. Transient VPg-free nascent RNA chains would not have been detected in the experiment described in Chapter III. Some nascent molecules might never receive covalently attached VPg, and if they comprised only a very small portion of all nascent chains at 3.5 h after infection, their 5' end structures might have gone undetected. However, since there is, as yet, no evidence to the contrary, the interpretation can be made that VPg is attached to all nascent positive strands at some time very early in their synthesis. The demonstration of a primer role for VPg may develop from in vitro studies of initiation of poliovirus RNA synthesis.

C. A Proposed Role for VPg and HeLa Unlinking Enzyme in Poliovirus RNA Processing

Since polio polysomal RNA lacks VPg and terminates with the same nucleotide sequence as virion RNA, it appears that VPg is removed from some newly made polio positive strand molecules. HeLa unlinking enzyme could perform the necessary specific cleavage to remove VPg. However, it is possible that this removal of VPg is actually not necessary for
the subsequent functions of a newly made viral RNA molecule. VPg may be gratuitous for translation of polio RNA. Furthermore, covalently bound VPg may not be necessary for encapsidation; its presence on virion RNA may simply reflect the relative kinetics of encapsidation and VPg removal. However, it is more attractive to consider that VPg may be removed rapidly from a newly made poliovirus RNA molecule, and that the presence of VPg on virion RNA has functional significance. If covalently bound VPg is a requirement for packaging of an RNA molecule, then removal of VPg would serve to direct new molecules to other functions. Those molecules with VPg removed would become mRNA or serve as template for the synthesis of negative strands. Those molecules retaining VPg would be packaged into virions. 5' terminal VPg may form part of a structure at the 5' end of positive strands which can specifically bind to procapsid and thereby initiate packaging. This association between procapsid and VPg may protect the VPg-RNA linkage from cleavage by unlinking enzyme. This model would explain why no virion RNA lacks VPg and also provides the basis for a kind of feedback control of polio mRNA production.

Early in infection it is undoubtedly advantageous for poliovirus to amplify viral mRNA and to thereby synthesize maximal amounts of polio-specific proteins, including the replicase molecule and other products essential for viral RNA and protein synthesis; encapsidation of viral RNA at this very early stage is of lesser importance. Before appreciable quantities of procapsid have been synthesized and assembled, most nascent and newly made positive strands would be free of bound procapsid. VPg could therefore be easily removed by unlinking activity. The bulk of new polio positive strands would thus be free to participate in protein synthesis and RNA replication. This would facilitate the expression of
viral functions such as polio replicase, host shut-off, and accumulation of virion proteins.

Later in infection, say from 3-5 h after infection began, packaging of RNA into virions would be of primary importance and accumulation of new mRNA unnecessary. The presence of large quantities of viral proteins and consequent rapid assembly of procapsid would result in the binding of procapsid to most, if not all, nascent positive strands. As postulated above, this binding would prevent the removal of VPg by unlinking enzyme, thereby preventing further accumulation of mRNA, and at the same time, would commit each new positive strand to eventual encapsidation into a mature virion. This model is consistent with the fact that the fraction of viral RNA which is encapsidated increases as infection proceeds (Baltimore, 1969b). The hypothesis that polio RNA containing VPg specifically binds to procapsid, and RNA lacking VPg does not, may be testable in vitro.

According to the above model, HeLa unlinking activity plays an important role in poliovirus replication. The basic similarities among the replicative strategies of all picornaviruses implies that unlinking activity may also be involved in other picornavirus infections. Unlinking enzyme is found in cells of diverse origins (Chapter III), a fact consistent with the diversity of picornavirus natural hosts (Cooper et al., 1978).

3) **NORMAL CELLULAR FUNCTION OF HEla UNLINKING ENZYME**

Since HeLa unlinking activity is found in uninfected cells, it was assumed that this enzyme has some function not normally related to poliovirus infection. The uridylyl-removing enzyme of E. coli (Adler, et al., 1975) cleaves the tyrosine-phosphate bond between UMP and PII protein.
HeLa unlinking activity is probably not the analogous eukaryotic enzyme, for it does not remove UTP from VPg. Unlinking enzyme could, however, normally remove a covalently bound nucleic acid polymer from protein.

The occurrence of 5' terminal protein on diverse RNA and DNA viruses suggests that such linkages may be common and may also be found on cellular nucleic acid. Since terminal protein of some of these viruses may play a role in RNA or DNA synthesis, then by analogy, cellular protein-nucleic acid linkages may be associated with cellular RNA or DNA synthesis. Cellular unlinking activity may normally function to cleave those linkages at some appropriate stage of the synthetic process.

By analogy to the E. coli uridylyl-removing enzyme mentioned above, which also catalyzes the reverse (uridyl-transferase) reaction (Adler et al., 1975), unlinking enzyme may normally participate in the attachment of a protein to the 5' end of a polynucleotide chain. Such a 5' blocked polynucleotide might be an intermediate in a synthetic or degradative process, or a ligation.

The speculative nature of the above discussion points to the need to learn more facts pertaining to the normal function of unlinking enzyme. A more precise definition of the substrate specificity of this enzyme is required. Synthetic analogs of the structures comprising the 5' terminal protein-nucleic acid linkage of poliovirus could be tested for sensitivity to cleavage by the purified enzyme or for inhibiting effects on the enzyme activity. If a specific inhibitor of the enzyme could be found or designed, the role of this enzyme in polio infected or in uninfected cells might be investigated biochemically. If unlinking enzyme is essential for successful polio infection, then a mutant in the cellular gene for the enzyme would be resistant to polio infection.
Isolation of cell mutants temperature sensitive for resistance to polio infection might yield a cell line temperature sensitive for unlinking activity. Such a cell would be useful for the study of that enzyme.
BIBLIOGRAPHY


