THE REGULATION OF RNA AND PROTEIN SYNTHESIS IN POLIOVIRUS-INFECTED CELLS

All the work reported in this thesis was done by myself except for the preparation of some of the 
radio-labelled proteins and some analyses of polypeptides in sucrose
gradients. These were done in collaboration with
Dr P.D. Cooper and are indicated in the text.

By

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STATEMENT

All the work reported in this thesis was done by myself except for the preparation of most of the $^{125}$I-labelled proteins and some analyses of polypeptides in sucrose gradients. These were done in collaboration with Dr P.D. Cooper and are indicated in the text.

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THESIS SUMMARY

The work described in this thesis was aimed towards an understanding of the role of viral polypeptides in the control of viral replication and translation, and in the inhibition of host RNA and protein synthesis following infection of cells with poliovirus. Chapter One reviews the background literature which led to the experimental work described in the remaining chapters. The approaches taken and the main findings were as follows.

(1) The small ribosomal subunit was examined for the presence of viral protein which could be responsible for the inhibition of host protein synthesis and/or the initiation of viral specific protein synthesis. The viral polypeptides VP0, VP1, VP3, were found apparently attached to this subunit. These same polypeptides were discovered in complexes with the density of viral mono- or polyribosomes.

(2) The site of viral RNA replication in the cell, the replication complex, was also examined and found to contain, among other viral proteins, VP0, VP1 and VP3. The detection of VP0, VP1, VP3 in both ribosomal and replicative structures within infected cells is presented as evidence supporting the equestron model. The main hypothesis of this model is that there exists a regulator of viral replication, translation and maturation.

(3) DNA-dependent RNA polymerase activity was measured at both high and low ionic strength in isolated nuclei and confirmed to be reduced in nuclei from infected
cells. It was also reduced in aggregate enzyme, a crude chromatin preparation derived from lysed nuclei. The polypeptide composition of aggregate enzyme was determined by polyacrylamide gel electrophoresis, but no viral proteins were detected.

(4) A small plaque mutant and three cold sensitive mutants were isolated from a mutagenized stock of the ts⁺ strain of poliovirus. This type of mutant is particularly likely to have a defect in regulatory functions, e.g. the control of host or viral macromolecular synthesis. The work did not advance sufficiently for this proposal to be tested, and this thesis simply reports the isolation of this type of mutant.

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1.5 Implications - The Experimental Approaches
The sequence of events following the infection of cells with poliovirus are well documented. The temporal relationship of viral RNA replication, viral translation, the inhibition of host RNA and protein synthesis, and virion formation has been confirmed in many laboratories and yet the control mechanisms acting on these aspects of the poliovirus growth cycle have remained obscure. The experimental work of this thesis was directed towards an understanding of the role of viral polypeptides in regulating these processes.

Chapter One reviews the possible functions of poliovirus proteins and their relation to the genetic map of temperature sensitive mutants. The reasons for seeking specific regulatory viral proteins are given. The poliovirus-induced inhibitions of host cell protein and RNA synthesis are described in the light of recent advances in understanding transcription and translation in normal cells. In the final section of this chapter the reasons for the experimental designs are summarized.

1.1 Gene Function in Poliovirus

The poliovirus genome is a single strand of RNA of molecular weight $2.6 \times 10^6$ daltons (Granboulan and Girard, 1969; Tannock, Gibbs and Cooper, 1970), corresponding to a total coding potential of approximately 2,500 amino acids or 270,000 daltons of protein (Cooper, Geissler, Scotti, and Tannock, 1971). However, the cistrons of the poliovirus genome are not translated individually, but rather the RNA is translated into one large polypeptide which is then
progressively cleaved into polypeptides of lower molecular weight (Summers and Maizel, 1968; Holland and Kiehn, 1968; Jacobson and Baltimore, 1968a). The viral polypeptides are detected by labelling infected cells with radioactive amino acids at a time when the virus has induced the repression of host cell protein synthesis, and then analysing the radioactive polypeptides by gel electrophoresis.

Besides the four virion proteins, the cytoplasm of infected cells contains viral polypeptides of molecular weights from 130,000 daltons to 5,000 daltons, the relative amounts depending on the time of labelling. Very large polypeptides (up to about 250,000 daltons) can be detected either in vivo in the presence of amino acid analogues, which presumably interfere with the cleavage mechanism when incorporated into protein (Jacobson, Asso, and Baltimore, 1970; Baltimore et al., 1971), or in vitro in a protein synthesizing system of membrane-bound poliovirus-specific polyribosomes (Summers, Roumiantzeff and Maizel, 1971). First reports put the total number of viral polypeptides at 14 (Summers, Maizel and Darnell, 1965) but recently the number has risen to 25 - 30 with the use of autoradiography of gels (Abraham, personal communication). Some of the polypeptides may only be intermediates in the cleavage process or degradation products, and have no other function. Some almost certainly have important functions in, for example, RNA-dependent RNA polymerase activity, repression of host cell macromolecular synthesis and perhaps maturation of the virus and cytopathic effects. Despite the
Figure 1 Genetic map of poliovirus (Cooper et al., 1971). The numbers represent ts mutants, above the line as published by Cooper (1968), and below the line as added since then; g, g', m and S-7 represent loci for resistance to guanidine, dextran sulphate and S-7, respectively; DS.RNA = defect in ability to make double-stranded RNA at 39.5°C; SS.RNA = defect in ability to make single-stranded RNA at 39.5°C; ΔH = labile infectivity at 45°C; cy = cystine dependence; pti = defect in ability to prevent host thymidine incorporation at 39.5°C. The corresponding symbols with a plus sign (e.g. DS. RNA+) denote no difference from wild type. As discussed in the text, the available evidence suggests that the 3'-5' orientation is left-right.
difficulties introduced by post-translational cleavage, some progress has been made in determining which polypeptides have functional roles. These will be briefly discussed now as a background to the work described in this thesis and the equestron model (see Chapter Six).

(a) Genetic analysis

The information obtained from the genetic map of temperature sensitive mutants of poliovirus has been reviewed by Cooper (1969) and Cooper et al. (1971) and is partly summarized in Figure 1. Mutants affecting viral coat protein occupy 48% of the genetic map, and virion protein contains a unique sequence of approximately 1000 amino acids, deduced from amino acid analyses and the number of tryptic peptides in virus labelled with cystine, histidine or methionine (Cooper and Bennett, 1973). Since this corresponds to 40% of the total amino acids coded, it seems that assuming a random distribution of mutants the genetic map covers at least 70 - 80% of the genome (Cooper et al., 1971).

The remaining temperature sensitive mutants affect the synthesis of viral RNA. Those at the opposite end of the map to the coat protein genes synthesize neither double-stranded nor single-stranded RNA at the restrictive temperature. Mutants nearer the coat protein region synthesize double-stranded RNA only (Cooper, Stanček and Summers, 1970; Cooper et al., 1971). These findings led to the proposal that there are two polymerase enzymes or enzyme factors for synthesizing (a) the RNA strand complementary to
the infecting strand, and (b) more infectious or viral RNA with the complementary RNA as template.

Taken together, mutants affecting viral RNA synthesis and coat protein occupy 0.916 map units of the total 1.08 units (Cooper et al., 1971). The only space left for a gene coding for some other viral function is beyond the ends of the known map, or between the polymerase and protein genes where there is a small gap bounded by ts-150 and ts-22 and capable of specifying a protein of perhaps 30,000 daltons. However it is possible that the gap is also part of a polymerase and/or coat protein gene.

Two types of experiments designed specifically to induce mutations at the 3' end or 5' end of poliovirus RNA, followed by genetic mapping, suggested that the polymerase genes were at the 3' end and the coat protein genes at the 5' end of the genome (Cooper et al., 1971). This conclusion was supported by experiments with pactamycin, an inhibitor of the initiation of protein synthesis. By pulse labelling infected cells after the addition of pactamycin, polypeptides coded by genes towards the 3' end of the RNA should be more heavily labelled than their 5' counterparts. The results implied that NCVP1, the precursor to virion proteins (see below), is coded by genes near the 5' end of the RNA, and that the gene for NCVP2, a candidate for a polymerase protein (see below) is near the 3' end (Summers and Maizel, 1971; Taber, Rekosh and Baltimore, 1971).
(b) Polymerase proteins

A smooth membrane fraction from infected cells, rich in viral RNA polymerase activity, was obtained by centrifugation in discontinuous sucrose gradients (Caliguiri and Tamm, 1970a, 1970b; Caliguiri and Mosser, 1971). After deoxycholate treatment, pulse labelled RNA and viral RNA polymerase activity sedimented at approximately 155S (Caliguiri and Mosser, 1971). Gel electrophoresis of labelled proteins from this region demonstrated the presence of a polypeptide of similar molecular weight to VP1, possibly NCVPX (Jacobson and Baltimore, 1968a; Jacobson et al., 1970) as well as VP0, VP1, VP3 and other polypeptides of higher molecular weight. Unfortunately a gel of total cytoplasm for the same experiment was not given, and thus it is difficult to judge the degree of selective enrichment of polypeptides in the 155S fractions. The results of Cooper et al. (1971) using a similar membrane fraction implicated NCVP1\(^{1/2}\) or NCVP2 (80,000 - 95,000 daltons) and NCVP9 (8,000 - 10,000 daltons) as possibilities for polymerase proteins. NCVP2 was found to be a relatively stable protein in pulse chase experiments (Summers and Maizel, 1968; Summers et al., 1971), suggesting it may have some functional role other than as an intermediate in the cleavage process.

The conclusion from the polymerase work is that several viral polypeptides are candidates for polymerase proteins, but none is certainly identified.
(c) **Structural proteins**

The likely precursor to the virion proteins (VP1, VP2, VP3 and VP4) has been identified as NCVPl (molecular weight 105,000 - 130,000 daltons). The similarity of a tryptic digest of this polypeptide to that of purified virion protein compared by ion exchange chromatography suggested such a relationship (Jacobson *et al.*, 1970; Summers *et al.*, 1971). Tryptic digests of NCVP2 and virion protein were obviously dissimilar, although several peptides were common to both. Furthermore, a comparison of the tryptic peptides of VP0 (NCVP6) with that of VP2 and VP4 supported the hypothesis that VP2 and VP4 are derived from the cleavage of VP0 (Jacobson and Baltimore, 1968b).

The gene order for the coat protein region of the genome has been studied using pactamycin as described above, and emetine, a drug which prevents the release of nascent polypeptides from polyribosomes (Grollman, 1968). A brief period of labelling followed by emetine treatment will permit the release of only a few radioactive NCVPl molecules, more heavily labelled in the region coded by nucleotide sequences towards the 3' end of the RNA. Rekosh (1972) compared the amount of radioactivity in the individual proteins of virus obtained six hours after infection from cells which three hours earlier had (a) the pactamycin or (b) the emetine treatments as described above, or (c) label with no drug. The distribution of radioactivity between the virion proteins suggested a gene order from the 5' to the 3' end of the genome of VP4, VP2,
VP3, VP1, assuming that the relative proportions of the proteins selected for virion assembly were the same as the relative proportions synthesized during the labelling period.

(d) **Assembly of virus**

The cytoplasm of infected cells contains empty capsids, which sediment at approximately 70S in sucrose gradients (Maizel, Phillips and Summers, 1967). They are not degradation products of virions since they contain VP1, VP3, little or no VP2, no VP4, and a major polypeptide VP0 (NCVP6) which has a larger molecular weight than any virion protein. Evidence that VP2 and VP4 are derived by cleavage from VP0 was mentioned above. Jacobson and Baltimore (1968b) suggested that this change occurs when virions are formed as a result of the association of viral RNA with the empty capsids. They found that a structure sedimenting at 74S accumulated in infected cells in the presence of guanidine. On removal of guanidine, the amount of virus increased with a concomitant decrease in the quantity of 74S material. However it is not certain that the structures of 74S were empty capsids, or that they were the immediate protein precursors to virions.

A particle sedimenting at 14S, also containing VP0 (NCVP6), VP1 and VP3 as the major polypeptides, was shown to be capable of assembly into 73S particles with a polypeptide composition similar to empty capsids (Phillips, Summers and Maizel, 1968). The 14S particles self-assembled at 37°C in a reaction that was concentration-
dependent (Phillips, 1971). They also assembled at lower concentrations in the presence of an extract of infected but not uninfected cells (Phillips, 1969). In addition, the self-assembled 73S particles contained structures like empty capsids when examined in the electron microscope (Phillips, 1971). Whether the factor promoting assembly in infected cell extracts is a viral protein is uncertain. Since its activity is abolished by typsin and deoxycholate, it is probably associated with membranes (Phillips, 1969; Phillips, 1972).

(e) The need for regulation during viral growth and the equestron model

The necessity to postulate a regulator during the poliovirus growth cycle is discussed by Cooper, Steiner-Pryor and Wright (1973). Briefly, the facts which implicate a regulator are as follows:

(a) Almost half of the viral protein and RNA synthesized is converted into virions and thus the maturation process is reasonably efficient (Penman, Becker and Darnell, 1964; Baltimore, Girard and Darnell, 1966; Baltimore, 1969).

(b) The mature virion contains viral RNA and protein gene copies in the ratio of 1:60 (Horne and Nagington, 1959; Cooper and Bennett, 1973), although RNA can be made more rapidly from an RNA template than protein. About nine new RNA molecules can be made each minute from an RNA template (Baltimore, 1969), but only three to four protein copies can be synthesized in this time (Summers,
Maizel and Darnell, 1967; Rekosh, 1972). Nevertheless protein and RNA are actually made in the correct 60:1 ratio (Darnell et al., 1961).

(c) At midcycle, three to four ribosomes per minute are being added to the 5' end of viral RNA. If the affinity of ribosomes for the 5' end of viral RNA was as large as this early in infection, the synthesis of complementary RNA, which proceeds in the opposite direction to protein synthesis and takes approximately one minute (Baltimore, 1969), would never be completed. Therefore, although the affinity of ribosomes for the host messenger RNA apparently decreases after infection (Leibowitz and Penman, 1971), the affinity of the ribosomes for viral RNA seems to increase during the growth cycle.

(d) Experimentally, the doubling time of viral RNA is about fifteen minutes (Cooper, 1964a; Baltimore et al., 1966), although the theoretical minimum possible is less than two minutes (Baltimore, 1969). Furthermore, viral RNA and complementary RNA are not made in equal proportions, but with a 5:1 ratio (Noble and Levintow, 1970; Bishop and Levintow, 1971).

Both the ribosomal affinity changes [(c) above] demand modifiers attached to, or removing something from, the ribosome. The most economical case is for these modifiers to be the same, and to be viral proteins specific for the small ribosomal subunit rather than for a
variety of messenger-specific initiation factors. To promote binding of the 45S subunits and viral RNA, the modifier should also have an affinity for the 5' end of viral RNA. A modifier for the 5' end of viral RNA could also restrict the release of complementary RNA from its viral RNA template and cause the slow doubling time of viral RNA [(d) above]. If the 5' modifier is viral protein, then genetic economy [section 1.1 (a)] requires that it and the ribosomal modifier are the same.

However, a 5' modifier restricting replication would also hinder maturation, and so would need removal later in the cycle. Since all gene products increase equally (Summers et al., 1965), simple competition between structural units and modifier would not suffice to remove it. Consequently, the modifier is likely to be labile with a half-life between the viral RNA doubling time of 15 minutes (Cooper, 1964a; Baltimore et al., 1966) and the time occupied by maturation, i.e. 30 - 60 minutes (Penman et al., 1964; Baltimore, 1969).

Thus Cooper et al. (1973) propose a hypothetical poliovirus regulator with affinities for some ribosomal factor or subunit and for the 5' end of viral RNA. To promote binding of viral RNA and ribosomes, and to conserve genetic information, it is very likely to be a single particle, binding to the small ribosomal subunit but with a half-life of 20 - 40 minutes. This particle has been termed the equestron. The possible candidate for the equestron is discussed in Chapter Six.
1.2 Protein Synthesis in Virus-Infected Cells

(a) Inhibition of host cell protein synthesis by picornaviruses

The inhibition of synthesis of macromolecules in cells infected with animal viruses has been reviewed by Roizman and Spear (1969), and more specifically in cells infected with picornaviruses by Martin and Kerr (1968). In this section I shall deal mainly with the effects on protein synthesis by infection of cells with poliovirus. Other relevant data for the picornaviruses will also be discussed.

In HeLa cells infected with poliovirus, cellular protein synthesis declines within an hour of infection (Zimmerman, Heeter and Darnell, 1963; Holland and Peterson, 1964). Inhibition of host cell protein synthesis by infection has been noted with a number of other picornaviruses including Mengovirus in L cells (Baltimore, Franklin and Callender, 1963), Maus Elberfeld (ME) virus in L cells (Hausen and Verwoerd, 1963), Encephalomyocarditis virus (EMC) in Krebs II ascites cells (Martin, Malec, Sved and Work, 1961), and Foot-and-mouth disease virus in BHK 21 cells (Brown, Martin and Underwood, 1966). A more complete list is given by Martin and Kerr (1968).

There is evidence suggesting that the virus-induced inhibition of host cell protein synthesis is not simply a result of the inhibition of RNA synthesis which also occurs in these cell-virus systems (see later). Firstly, the rate of host cell protein synthesis declines
more rapidly than the rate of cellular RNA synthesis (Zimmerman et al., 1963; Bablanian, Eggers and Tamm, 1965). Secondly, protein synthesis declines and polysomes disaggregate faster after infection than do the same in uninfected cells treated with actinomycin D to prevent the synthesis of new messenger RNA. This has been observed in HeLa cells infected with poliovirus (Penman, Scherrer, Becker and Darnell, 1963), and in Krebs II ascites cells infected with EMC virus (Dalgarno, Cox and Martin, 1967). The rate of inhibition of cellular protein synthesis is also partly determined by the type of host cell. Poliovirus causes a more rapid inhibition in HeLa cells than in HEL or ERK cells (Bablanian et al., 1965). Mengovirus inhibits protein synthesis more rapidly in L cells than in HeLa cells (McCormick and Penman, 1967). Mengovirus also inhibits protein synthesis in one line of Novikoff rat hepatoma cells, but not another (Plagemann and Swim, 1966).

An important question is whether some component(s) of the infecting virions is the primary cause of the inhibition or whether newly made RNA or protein is responsible. The evidence available suggests that protein synthesis is required after infection. Penman and Summers (1965) infected HeLa cells with poliovirus in the presence of cycloheximide, an inhibitor of protein synthesis. When the infected cells were washed free of cycloheximide, the virus-induced inhibition of protein synthesis was not immediate, but only became apparent after thirty minutes of
protein synthesis. The amino acid analogue p-fluorophenylalanine (FPA) prevented the virus-induced inhibition of protein synthesis in L cells infected with Mengovirus (Baltimore, Franklin and Callender, 1963) and with ME virus (Verwoerd and Hausen, 1963). Inactivation of poliovirus with ultraviolet light prior to infection also abolished the inhibition of cellular protein synthesis (Penman and Summers, 1965). If one assumes that the irradiation specifically inactivated the RNA of the virus, then it seems that a virus-coded protein is necessary for the inhibition. This protein is apparently active in small amounts as host protein synthesis is still repressed in the presence of guanidine, an inhibitor of viral growth (see below).

Objections to the likelihood of the inhibitory protein being virus-coded have been raised by Martin and Kerr (1968). (a) The concentration of FPA used by Baltimore, Franklin and Callender (1963) to demonstrate the ability of the drug to prevent virus-induced inhibition was one hundred times that which will essentially stop viral protein synthesis (Baltimore and Franklin, 1963). (b) If L cells were treated with interferon before infection with Mengovirus, the virus-induced inhibition of cellular protein synthesis was unaffected (Levy, 1964). However, neither of these objections could be maintained if a small amount of virus-coded protein was sufficient to inhibit host protein synthesis, and the effect of guanidine (see above) suggests that this is indeed the case. In both (a)
and (b) a small amount of virus "leak" could explain the results. In addition, if the inhibitory protein was host-coded, it would be necessary for the host messenger RNA coding for the protein to be made before infection, since the virus-induced inhibition occurs in the presence of actinomycin D (Penman and Summers, 1965).

The work of Hunt and Ehrenfeld (1971) suggested that the double-stranded RNA present in cells infected with poliovirus may cause the inhibition of cellular protein synthesis. Double-stranded poliovirus RNA inhibited the initiation of protein synthesis in rabbit reticulocyte lysates, as did double-stranded reovirus RNA and synthetic copolymers, at extremely low concentrations (Ehrenfeld and Hunt, 1971). However there is no direct evidence that double-stranded poliovirus causes the inhibition in vivo, and in addition the mutant ts-20 which makes no detectable double-stranded RNA at the restrictive temperature of 39.5°C (Cooper, Stanček and Summers, 1970) is not defective in its ability to repress host cell protein synthesis at this temperature (Steiner-Pryor and Cooper, unpublished results).

Whatever the inhibitor, RNA or protein, it is made in the presence of guanidine in poliovirus-infected cells (Penman and Summers, 1965; Bablanian et al., 1965). Guanidine used at a similar concentration in uninfected cells has no effect on protein synthesis, but in infected cells prevents detectable synthesis of viral RNA and the burst of viral protein synthesis starting approximately 2.5 hours
after infection (Crowther and Melnick, 1961; Caliguiri and Tamm, 1968a; Baltimore, 1968, 1969). It is possible that limited replication or translation of the input genomes could produce sufficient inhibitor. In support of this idea, increasing the multiplicity of infection results in a more rapid cessation of cellular protein synthesis (Penman and Summers, 1965).

Thus it seemed probable, from a review of the literature when the work of this thesis was started, that the inhibitor of cellular protein synthesis in poliovirus-infected cells was a viral protein made after infection. The other possibilities were that the inhibitor was some component of the infecting virion, a host-coded protein, or double-stranded RNA. Although each had some arguments in its favour, none of these alternatives seemed very likely.

(b) Polyribosomes in poliovirus-infected cells

When HeLa cells infected with poliovirus are incubated in the presence of actinomycin D and guanidine for two hours, the number of host cell polysomes (with an average sedimentation coefficient of 200S) is reduced by approximately 75% (Summers et al., 1965). If the cells are then washed free of guanidine (reversal), host cell polysomes are not detectable after a further incubation of one hour. Two hours after reversal, virus proteins can be resolved by gel electrophoresis and the cells possess large virus-specific polysomes (380S) containing viral RNA (Summers and Levintow, 1965), and approximately thirty-five ribosomes per polysome (Summers,
Maizel and Darnell, 1967). Between one and four hours after reversal over 90% of protein synthesis is viral specific (Summers, et al., 1965). At four to five hours after reversal, the polysomes decrease in size to about 200S as the number of ribosomes per polysomes is reduced to approximately twenty, but the size of the messenger RNA is unchanged (Summers et al., 1967).

The disappearance of host cell polysomes following infection of poliovirus has been examined by Leibowitz and Penman (1971). When cells are infected in the presence of actinomycin and guanidine, the average sedimentation coefficient of host cell polysomes decreases within one hour. The rate of translation and the size of the polyribosome-associated messenger RNA are unchanged, nor do the ribosomes apparently detach prematurely from the message. Thus Leibowitz and Penman suggest that the initiation of protein synthesis is inhibited. The ribosomes of infected cells do not translate host cell messenger RNA despite its continuing presence (Willems and Penman, 1966), but are capable of initiating polypeptide synthesis on viral RNA as messenger. An obvious way in which the specificity of initiation could be changed is by an alteration in the specificity of the initiation factors for protein synthesis. This could be effected by a change in the relative amounts of host-coded initiation factors, or by a virus-coded protein acting directly as an initiation factor.
(c) **Initiation of protein synthesis in bacterial and eukaryotic systems**

At this point it is appropriate to discuss the current knowledge of the initiation of bacterial and mammalian protein synthesis. More details are known for the bacterial systems but much can be extended to mammalian systems, which are of course more relevant to this thesis. Recent reviews on this topic have been written by Lucas-Lenard and Lipmann (1971), and Lengyel and Söll (1969).

In bacterial systems, the initiation of protein synthesis first involves the attachment of messenger RNA to a small ribosomal subunit in the presence of Mg\(^{++}\) and initiation factor IF3. The next step, requiring GTP, initiation factors IF1 and IF2, is the attachment of formylmethionyl-tRNA\(_f\) in response to the AUG codon forming the initiation complex. The initiation factors seem to be localized on the small subunit (Parenti-Rosina, Eisenstadt and Eisenstadt, 1969; Miller, Zasloff and Ochoa, 1969); factors IF1 and IF2 promote the AUG-dependent binding of the formylmethionyl-tRNA\(_f\) to the initiation complex (Salas et al., 1967), but IF3 is needed for the specific recognition of natural messenger RNAs (Revel, Herzberg and Greenshpan, 1969; Wahba et al., 1969). The large subunit then binds to the complex and translation proceeds.

The initiation of protein synthesis in eukaryotes similarly involves the interaction of the small
ribosomal subunit with an AUG triplet in messenger RNA, and the binding to the complex of a molecule of methionyl-tRNAf (Heywood, 1970; Smith and Marcker, 1970; Brown and Smith, 1970; Hoerz and McCarty, 1971). The formation of the complex in a rabbit reticulocyte cell free system depends on GTP and at least three factors (from an 0.5 M KCl ribosomal wash) similar in function to the initiation factors of bacterial cells (Prichard, Gilbert, Shafritz and Anderson, 1970; Shafritz and Anderson, 1970; Lubsen and Davis, 1972; Woodley, Chen, Bose and Gupta, 1972). Which mammalian factor has the same function as the bacterial IF3 in recognizing natural messenger RNA has not been determined with certainty (Crystal, Shafritz, Prichard and Anderson, 1971). However, the high salt ribosomal wash fraction of BHK cells infected with Foot-and-mouth disease virus (FMDV) stimulates FMDV-RNA dependent protein synthesis in vitro (Ascione and Vande Woude, 1971), and the binding of Rauscher leukemia virus (RLV) RNA to ribosomes needs factors derived from the high salt wash of ribosomes from an RLV-infected cell line obtained from Balb/c mice (Wang, Naso and Arlinghaus, 1972). Thus it seems reasonable to propose that virus-specific initiation factors may exist in poliovirus-infected cells.

(d) T4-induced modification of E. coli ribosomes

The similarity of the steps in the initiation of protein synthesis in prokaryotes and eukaryotes makes it likely that some of the features of phage-infected bacterial cells will prove relevant to mammalian cells
infected with animal viruses. Before describing the limited work which has been done on the formation of viral polyribosomes in poliovirus-infected cells, the more detailed findings on the initiation of protein synthesis in phage-infected *E. coli* cells will be discussed, including possible changes in the initiation factors.

Hsu and Weiss (1969) first reported that after the infection of *E. coli* by phage T4, the ribosomes showed reduced amino acid incorporating activity with MS2 and *E. coli* template RNAs, but not with T4 RNA templates. Washing the ribosomes of infected cells with high salt released some factor(s) which, when mixed with washed ribosomes from uninfected cells, conferred on them the same selectivity in the translation of RNA templates. However the results implied that not all of the T4-induced inhibitory factor(s) were removed by high salt. If chloramphenicol was added before infection, the ribosomes of infected and uninfected cells exhibited similar activity with the three RNA templates.

Dube and Rudland (1970) showed that a messenger selection process functioned in infected cell extracts at the point of polypeptide chain initiation. They tested the ability of R17, f2, T4 and *E. coli* RNAs to act as messengers by measuring the ribosomal binding of formylmethionyl-tRNA₅ on nitrocellulose filters. T4 RNAs formed complexes with all permutations of salt-washed ribosomes and factors from uninfected and T4-infected cells, but almost none were formed with the other RNAs in the
presence of factors from infected cells. Not only was the total level of R17 initiation sites bound to the ribosomes decreased, but the proportion of the three R17 sites recognized was altered to favour the A site (Steitz, Dube and Rudland, 1970).

Thus the change in template specificity appeared to result almost exclusively from changes within the ribosomal or initiation factor fraction derived by washing the ribosomes in high salt. In particular, the factor IF3, which is needed for natural messenger RNA binding to ribosomes, was isolated from uninfected E. coli and separated into at least two species. One recognized late T4 RNA, the other recognized early T4 and MS2 RNA (Pollack et al., 1970; Berissi, Groner and Revel, 1971; Lee-Huang and Ochoa, 1971). The last mentioned workers found that in E. coli infected with T4, the factor responsible for the recognition of late T4 messenger sites was still present, whereas the corresponding IF3 factor for MS2 RNA was not detected.

In contrast to these findings, Goldman and Lodish (1972) claimed that their results indicated no change in the specificity of initiation in E. coli cells infected with T4, although they did not dispute that there was some change in the initiation factors. They concluded that a non-specific decrease in the efficiency of the initiation factors occurs following infection, and that a change in the amounts of factors is not necessary for the control of T4 protein synthesis. Yoshida (1972) also
found that IF3 from uninfected *E. coli* promoted the binding of ribosomes to both early and late T4 messenger RNA.

More recently interference factors have been described that change the specificity of initiation factor IF3 towards messenger RNA. Groner, Pollack, Berissi and Revel (1972) found a factor which functions as a cistron selecting factor for MS2 and probably T4 translation. Lee-Huang and Ochoa (1972) reported two factors, one inhibiting the IF3 component which recognizes MS2, *E. coli* and early T4 RNA, and the other inhibiting the IF3 component recognizing late T4 RNA.

Thus the exact nature of change in IF3 components following infection of *E. coli* with T4 remains uncertain, but there is no doubt that some change occurs. If one accepts that there is an alteration in the specificity of IF3 after infection, two mechanisms are possible (Yoshida, 1972). Host factors could recognize early and late T4 messenger RNA but be selectively altered or inactivated to permit selection of late T4 RNA only. Evidence for this idea is given by the results of Lee-Huang and Ochoa mentioned above. The other possible mechanism is that host IF3 factors only recognize early T4 messenger RNA, and that a new factor specific for late T4 RNA is made after infection. Evidence for this proposal comes from the work of Ihler and Nakada (1971) who found that initiation factors obtained from uninfected *E. coli* promoted the binding of ribosomes to the light strand of T4 DNA, but when obtained from T4 infected cells, bound
ribosomes only to the heavy strand of T4 DNA. The light strand of T4 DNA contains the messenger sequences for early T4 genes, and the heavy strand the sequences for late T4 genes (Guha and Szybalski, 1968).

New proteins synthesized after T4 infection and attached to the ribosomes were demonstrated by pulse-labelling (Smith and Haselkorn, 1969). Furthermore, Dube and Rudland (1970) described new polypeptides in the ribosomal factor fraction and a major one attached to the salt-washed ribosomes. The relation of these proteins to the functional alterations discussed above is unknown. They may be new viral or modified host IF3 factors, but another possibility is that they inactivate all IF3 factors except those needed for the translation of T4 messenger RNAs. The objection to the latter hypothesis is that if there are a large number of IF3 factors for the wide variety of messengers in a cell, the inactivation mechanism would have to cover a large number of proteins (Pollack et al., 1970).

(e) The initiation of formation of polyribosomes in poliovirus-infected HeLa cells

The probable precursors to polyribosomes in poliovirus-infected HeLa cells have been identified on the basis of their rate of sedimentation through sucrose gradients and their density in caesium chloride gradients after fixation with glutaraldehyde (Huang and Baltimore, 1970). In these studies the cells were infected and labelled with [3H]uridine in the presence of actinomycin D.
so that the only labelled structures in the cells were those containing viral RNA.

At three hours after infection it was approximately ten minutes before newly added label was found in polysomes. The viral RNA was made in the replication complex which sedimented heterogeneously at about 250S and had a buoyant density of 1.44 g/cc (Girard, Baltimore and Darnell, 1967; Baltimore and Huang, 1968). If protein synthesis and the recruitment of viral RNA into polyribosomes was stopped by the addition of cycloheximide, viral RNA synthesis continued and species of densities 1.40, then 1.44 and 1.47 g/cc were found in the time sequence given. If cycloheximide was removed and replaced by guanidine, which prevents viral RNA being released from the replication complex (Baltimore, 1968), the amount of these species was reduced and polyribosomes with a density of 1.54 g/cc became labelled. The interpretation of these results was that the 1.40 component corresponded to vRNP (viral RNA complexed with protein, Baltimore and Huang, 1970), the 1.44 component was a vRNP-small ribosomal subunit complex and the 1.47 component was a vRNP-ribosome complex (Huang and Baltimore, 1970). The presence of cycloheximide apparently caused these polysome precursors to accumulate, while the blockage of their production by guanidine on removal of cycloheximide allowed the label to flow through to polysomes again. The theoretical densities of the 1.44 and 1.47 species using the formula of Perry and Kelly (1966) for RNA-protein complexes agree fairly well with the experimental results.
1.3 Host Cell RNA Synthesis in Picornavirus-Infected Cells

(a) The inhibition of cellular RNA synthesis by picornaviruses

Following the infection of HeLa, ERK or human embryonic lung cell types with poliovirus, the rate of RNA synthesis declines until approximately three hours after infection when virus-specific RNA synthesis can be detected (Zimmerman et al., 1963; Fenwick, 1963; Holland, 1963; Bablanian et al., 1965). In Mengovirus-infected cells, the time and rate of inhibition are at least partly determined by the host cell. McCormick and Penman (1967) confirmed that Mengovirus caused rapid reduction in RNA synthesis in L cells (Franklin and Baltimore, 1962) but found that inhibition in HeLa cells was slower, and more similar to that observed in poliovirus-infected HeLa cells. Plagemann and Swim (1966) cultured two strains of Novikoff rat hepatoma cells and found that Mengovirus caused a depression of cellular RNA synthesis in only one of them. Virus-induced RNA synthesis followed the same time course in both cell lines.

Fenwick (1963) using poliovirus-infected ERK cells and Homma and Graham (1963) using Mengovirus-infected L cells found no evidence for selective inhibition of ribosomal or transfer RNA synthesis. Vesco and Penman (1969) showed that the synthesis of all non-mitochondrial associated RNA was depressed in Chinese hamster ovary cells infected with Mengovirus, but that the synthesis of mitochondrial-associated RNA was completely insensitive to
viral infection. Other evidence for selective inhibition of RNA synthesis in poliovirus-infected HeLa cells was described by Darnell et al. (1967). One and a half hours after infection, the synthesis of 45S ribosomal precursor RNA had decreased more than the synthesis of other nuclear RNA. Maturation of the 45S precursor to new ribosomal subunits was also inhibited in infected cells.

(b) Nature of the inhibitor

Like the virus-induced inhibition of protein synthesis, it seems that new protein made after infection rather than a component of the infecting virions is responsible for the depression of cellular RNA synthesis. This conclusion depends on the following observations.

(a) Puromycin reduced the inhibition of RNA synthesis in Mengovirus-infected cells (Baltimore, Franklin and Callender, 1963). (b) Cycloheximide prevented the virus-induced change in the maturation of ribosomal subunits in poliovirus-infected HeLa cells (Darnell et al., 1967).

(c) p-Fluorophenylalanine reduced the depression of cellular RNA synthesis in L cells infected with Mengovirus (Baltimore, Franklin and Callender, 1963) or with ME virus (Verwoerd and Hausen, 1963).

Evidence for the inhibitory protein being virus-coded comes from the work of Franklin and Baltimore (1962) who found that Mengovirus inactivated by ultraviolet light did not inhibit host RNA polymerase after infection of L cells (see later). Similarly photoinactivation of poliovirus containing proflavine prevented the virus-
induced depression of RNA synthesis (Holland, 1964). Evidence for the possible involvement of a host-coded protein is less compelling and consists of the following facts. (a) The host cell partly determines the rate of decrease of RNA synthesis as described above. (b) The concentrations of puromycin and p-fluorophenylalanine needed to stop the Mengovirus-induced inhibition of RNA synthesis in L cells were much greater than the concentrations needed to stop viral protein synthesis (discussed by Martin and Kerr, 1968). (c) In L cells infected with Mengovirus, treatment with interferon delayed but did not prevent depression of RNA synthesis (Levy, 1964).

(c) DNA-dependent RNA polymerase activity in infected cells

The apparent inhibition of cellular RNA synthesis in picornavirus-infected cells is not the consequence of an increased breakdown of RNA (Martin et al., 1961; Franklin and Baltimore, 1962; Fenwick, 1964) or a decreased rate of entry of the labelled precursors of RNA into the cellular pools (Martin et al., 1961). However, the activity of DNA-dependent RNA polymerase assayed in "aggregate enzyme" (Weiss, 1960, 1968) or isolated nuclei from HeLa cells infected with poliovirus (Holland, 1962; Holland and Peterson, 1964) and L cells infected with Mengovirus (Baltimore and Franklin, 1962) is less than in uninfected cells. Balandin and Franklin (1964) described a cytoplasmic factor in L cells infected with Mengovirus that
could inhibit RNA polymerase activity in nuclei from uninfected cells and was sensitive to trypsin but not to ribonuclease. No such inhibitor was obtained from poliovirus-infected HeLa cells by Holland (1962) or by Ho and Washington (1971), although the latter did find an inhibitor, lipid in nature, which inhibited the RNA synthesis of uninfected cells in vivo.

Since isolated nuclei and extracted aggregate enzyme contain both DNA-dependent RNA polymerase and the DNA template, it is not clear whether the enzyme itself is inhibited as a result of infection, or whether the template potential of the DNA is reduced. Evidence casting doubt on the latter idea was obtained by Holland (1962), who showed that the ability of extracted DNA to prime RNA synthesis with E. coli RNA polymerase in vivo was the same for DNA from both infected and uninfected cells. Similarly deoxyribonucleoprotein from the nuclei showed no differences in priming ability as a result of infection. However, these results obtained in vitro using bacterial polymerase may not be comparable with the situation in vivo with mammalian polymerase, because of possible differences in the control of transcription in prokaryotes and eukaryotes.

There is evidence that the reduction in RNA polymerase activity is not due to an inhibition of synthesis of the enzyme, as treatment of uninfected cells with puromycin for six hours does not reduce the RNA polymerase activity to that found in infected cells.
Soluble RNA polymerase activity obtained after the sonication of cells (Holland and Peterson, 1964) was also unchanged by infection of the cells with poliovirus. Since all the experiments described above were done with crude cell preparations, the answer to the question of whether enzyme activity or template activity is inhibited by infection remains open.

(d) Mammalian DNA-dependent RNA polymerase: recent developments

During 1970 it became accepted that mammalian cell nuclei contain at least two different activities of DNA-dependent RNA polymerase (Novello and Stirpe, 1970; Goldberg, 1970; Goldberg and Moon, 1970). A third activity has also been reported (Roeder and Rutter, 1970; Lindell et al., 1970; Zylber and Penman, 1971). Different forms of polymerase A (Chesterton and Butterworth, 1971a) and polymerase B (Kedinger, Nuret and Chambon, 1971; Weaver, Blatti and Rutter, 1971) have been separated, and the molecular weights of polypeptides constituting the polymerase B forms estimated by electrophoresis (Weaver et al., 1971; Kedinger et al., 1971; Chesterton and Butterworth, 1971b). The class B or II polymerase enzymes are sensitive to α-amanitin and are found in nucleoplasm (Jacob, Sajdel and Munro, 1970; Roeder and Rutter, 1970). The class A enzymes are insensitive to α-amanitin and consist of one nucleoplasmic enzyme (Zylber and Penman, 1971; Roeder and Rutter, 1970) and two forms (polymerase Ia,
Ib) in the nucleolus (Jacob et al., 1970; Roeder and Rutter, 1970; Chesterton and Butterworth, 1971a).

No studies on the activity of DNA-dependent RNA polymerase in poliovirus-infected cells have been reported since these recent developments. Weaver et al. (1971) suggested that the regulation of eukaryotic and prokaryotic RNA polymerases may be similar on the basis of structural likeness, since they found that the two forms of polymerase II from both calf thymus and rat liver have subunits of molecular weights in daltons of (190,000) (150,000) (35,000) (25,000) and (170,000) (150,000) (35,000) (25,000), respectively. The core enzyme of bacterial RNA polymerase has the subunit structure $\alpha_2\beta\beta'$ where the molecular weights of $\beta'$, $\beta$, $\alpha$ and $\omega$ are respectively 165,000, 155,000, 39,000 and 10,000 (Burgess and Travers, 1970). The $\omega$ polypeptide is not obligatory for enzyme activity.

The other component of bacterial RNA polymerase is the $\sigma$ factor of molecular weight 95,000, which may be purified with the core enzyme depending on the purification procedure. The $\sigma$ factor increases the activity of purified enzyme by stimulating initiation, not elongation (Dunn and Bautz, 1969; Travers and Burgess, 1969). Despite initial reports of phage-specific sigma-like factors (Summers and Siegel, 1969; Travers, 1969, 1970), the evidence that $\sigma$ permits the core enzyme of E. coli to recognize specific sequences must be treated with caution (Burgess, 1971). However, the replicases of the RNA
bacteriophages Qβ and f2 do contain bacterial components (Kondo, Gallerani and Weissmann, 1970; Kamen, 1970; Fedoroff and Zinder, 1971). As the RNA-dependent RNA polymerase in poliovirus-infected cells has yet to be purified, the involvement of host RNA polymerase components in the viral enzyme as a possible cause of the decrease in cell polymerase activity cannot be excluded.

1.4 Cold Sensitive and Small Plaque Mutants

(a) Cold sensitive mutants

Conditional lethal mutants have been widely used in the genetic analysis of bacteria, bacteriophage and animal viruses. There are several reasons for this. Firstly, the mutations are possible in a majority, if not all genes regardless of function, a fact which theoretically allows complete mapping of a genome. Secondly, complementation tests to define genes on a functional basis are possible, and thirdly, the ability to maintain the mutants permits a physiological or biochemical study of these functions (Hayes, 1968).

Temperature sensitive mutants are an important class of conditional lethal mutants and have made a significant contribution in our understanding of biological regulatory mechanisms. They can be further divided into two categories: (a) those unable to grow at a high range of temperature (heat sensitive), and (b) those unable to grow at a low range of temperature (cold sensitive). The large majority of mutants studied have been of the heat sensitive variety (Hayes, 1968; Fenner,
1969). The normally accepted basis for the heat sensitive phenotype is the production of a heat-labile protein (Horowitz and Fling, 1953; Maas and Davis, 1952) or one briefly heat-labile at the time of synthesis (Edgar and Lielausis, 1964). Cold sensitive mutants have been isolated from E. coli (O'Donovan and Ingraham, 1965; Guthrie, Nashimoto and Nomura, 1969) and bacteriophages T1, ØX-174, T4, f2, λ (Christensen and Saul, 1966; Dowell, 1967; Scotti, 1968; Silverman and Valentine, 1969; Cox and Strack, 1971). Although poliovirus strains have been adapted to grow at temperatures higher and lower than normal (Dubes and Chapin, 1956; Lwoff, 1962), there have been no reports of using a mutagenized stock to isolate cold sensitive mutants. The molecular basis of cold sensitivity is uncertain, since a protein which is stable at 37°C for example is unlikely to be labile at a lower temperature. One possibility is that mutant allosteric proteins at a temperature lower than normal are unable to go through certain conformational changes needed for their normal function (O'Donovan and Ingraham, 1965). One might expect cold sensitive mutants to be preferentially found in genes whose products have a regulatory function subject to allosteric control. In this context it is interesting that the cold sensitive mutants of phages λ (Cox and Strack, 1971) and T4 (Scotti, 1968) are not distributed randomly throughout the genome but tend to be localized in certain genes.
(b) **Small plaque mutants**

Strains of poliovirus which give small plaques have been known for a number of years (Dubes, 1956; Sabin, 1956; Nomura and Takemori, 1960; Carp and Koprowski, 1962). For some mutants, plaque formation is dependent on the composition of the agar overlay (Dubes and Chapin, 1958; Takemori and Nomura, 1960). Others probably give small plaques because of a reduction in burst size (Carp and Koprowski, 1962).

Of particular interest is a comparative study of a small (S) and large (L) plaque variant of Mengovirus by Amako and Dales (1967). L-Mengovirus induced a more rapid lysis of infected cells and yet viral RNA synthesis followed the same time course in cells infected with either variant. Synthesis stopped earlier in cells infected with L-Mengovirus because of cell lysis, and hence the burst size of S-Mengovirus was six to ten times greater than that of L-Mengovirus. Mutants of poliovirus that replicate at the same rate as wild-type virus but are altered in their ability to lyse cells have not been reported.

1.5 Implications - The Experimental Approaches

The results and conclusions discussed in the preceding literature review provided the basis for the work described in the remainder of this thesis. This section summarizes the main reasons for the approaches used in seeking a further understanding of the control of RNA and protein synthesis in poliovirus-infected cells.
Firstly, consider the inhibition of host cell protein synthesis by infection. The available evidence suggested the following. (a) Viral protein made after infection was responsible. (b) Initiation of translation was the point at which protein synthesis was changed in poliovirus-infected cells. (c) T4 infection of *E. coli* induced changes in the initiation factors normally found attached to the small ribosomal subunit. (d) The process of the initiation of protein synthesis was fundamentally similar in bacterial and mammalian cells. Accordingly, the polypeptide composition of the native small ribosomal subunits of infected cells was analysed by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulphate, in order to detect polypeptides that were possibly responsible for the inhibition of host protein synthesis and/or the initiation of viral protein synthesis. These studies were subsequently extended to an examination of ribosome-viral RNA-protein complexes and replication complex by a combination of gel electrophoresis and isopycnic centrifugation in caesium chloride density gradients.

Secondly, consider the inhibition of host cell RNA synthesis by infection. It was most likely that virus-coded protein synthesized after infection caused the inhibition, and it was clear that DNA-dependent RNA polymerase activity also decreased. Hence aggregate enzyme, a preparation that displayed this reduced activity when prepared from infected cells and which was composed of DNA,
DNA-dependent RNA polymerase and other associated proteins, was analysed by gel electrophoresis for the presence of viral polypeptides.

Finally, there were adequate reasons for commencing a programme to isolate cold sensitive and small plaque mutants. The following possibilities were presented. (a) Gene functions not detected in other conditional lethal systems might be understood. (b) Mutants might be confined to certain genes, especially those whose gene products had a regulatory function.

2.4 Preparation of cytoplasmic extracts and their analysis on sucrose and cesium chloride gradients

(a) Preparation of cytoplasmic extracts and analysis on sucrose gradients

(b) Fixation with glutaraldehyde and cesium chloride gradients

(c) Determination of the absorbance at 260 nm of sucrose gradient fractions

(d) Determination of acid-insoluble radioactive activity in fractions from gradients

2.5 Preparation of radioactive virus and empty capsids

(a) Virus

(b) Top component or naturally occurring empty capsids

(c) Production of artificial empty capsids
CHAPTER TWO
MATERIALS AND METHODS

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2.9 Source of chemicals
2.1 Cell Cultures

(a) Growth of cells

The cells used for all the work described in this thesis were human amnion strain U cells (Pohjanpelto, 1961). They were routinely cultured in Eagle's medium containing double the concentration of amino acids and vitamins described by Eagle (1959) or in Auto-Pow, autoclavable powdered Eagle's medium (Yamane, Matsuya and Jimbo, 1968), supplemented with 10% calf serum, 1.25 mg/ml sodium bicarbonate, 146 μg/ml of L-glutamine and 100 μg/ml each of penicillin, streptomycin and neomycin. The cells were grown in 2.25 litre Winchester bottles, seeded with 16 x 10^6 cells and harvested five days later when the cell number was approximately 120 x 10^6. The bottles contained 200 ml of medium and were continuously rotated with the axis horizontal (1 revolution/12 minutes) at 37°C. Cells were removed from the glass by a 5 minute incubation at 37°C with 30 ml of a solution containing 0.025% trypsin, 0.02% ethylene diamine tetra-acetate (EDTA), 0.2 mg/ml NaHCO_3, 8 mg/ml NaCl, 0.4 mg/ml KCl, 60 μg/ml Na_2HPO_4 and KH_2PO_4. The cells were washed once with growth medium, suspended in the same medium at 6 x 10^6 cells/ml and maintained at 0°C until used.

Preparation of the media and the growth of cells was usually carried out by the media and tissue culture laboratories of this department. Eagle's medium was in use during the isolation of mutants and the DNA-dependent RNA polymerase work, while Auto-Pow was in use during the ribosomal protein experiments.
(b) Spinner cultures

Unless plaque assays or further monolayer cultures were prepared, the cells were stirred overnight at 37°C in suspension at $6 \times 10^5$ cells/ml in medium identical to that described above except that the Eagle's medium lacked calcium chloride, and for both Eagle's and Auto-Pow the quantity of calf serum and bicarbonate was halved. Cells from such a spinner were used in the majority of experiments to be described. In these experiments, cells were maintained at a concentration of $10^6$/ml if treated with guanidine and/or actinomycin D, and at $4 \times 10^6$/ml during labelling periods. The media contained 2% calf serum and 0.9 mg/ml sodium bicarbonate.

(c) Monolayer cultures

(i) Tubes: $2.5 \times 10^5$ cells in 1 ml of Eagle's medium (containing 10% calf serum, 10% tryptose phosphate broth, 1.4 mg/ml sodium bicarbonate and adjusted to pH 7.3 with 1 N NaOH) were added to flat-bottomed glass vials with an internal diameter of 17 mm. These were stoppered and incubated at 37.2°C overnight in an upright position. The monolayer of cells was confluent the following day.

(ii) Bottles: cell monolayers were also grown in 200 ml Kimax bottles containing 15 ml of the same medium. Seeding with $4.8 \times 10^6$ cells gave a confluent monolayer in 24 hours. The bottles were sealed with screw caps and then with plastic adhesive tape prior to immersion in a water bath at 37.2°C.
All strains were poliovirus type I; the Mahoney strain was used for the experiments in sections 3.3, 3.4 and 4.3, and the ts\(^+\) strain (Cooper, 1964b; Cooper, Johnson and Garwes, 1966) for those described in sections 3.2, 3.5, 4.2 and 5.2.

To prepare virus stocks, a cell suspension was stirred \((10^7 \text{ cells/ml})\) at \(0^\circ\text{C}\) for 1 hour with a virus suspension containing \(20 - 30 \text{ pfu/cell}\). The suspension was then diluted to \(2 \times 10^6 \text{ cells/ml}\) with spinner medium and incubated 6 hours at \(37^\circ\text{C}\) in a spinner culture. The whole culture was frozen and thawed, and the cell debris removed by centrifugation at \(10,000 \text{ g}\) for 10 minutes. The supernatant was dispensed in 10 ml portions and frozen at \(-15^\circ\text{C}\). Such a stock contained \(4-8 \times 10^8 \text{ pfu/ml}\). More concentrated stocks were obtained by pelleting the virus from this supernatant and centrifuging it to equilibrium in a caesium chloride gradient, as described later for the preparation of labelled virus [section 2.5 (a)].

Cells from an overnight spinner culture were routinely infected by stirring them in growth medium at \(0^\circ\text{C}\) with a magnetic bar at a concentration of \(1-5 \times 10^7 \text{ cells/ml}\) in the presence of virus at an input multiplicity of \(30 - 50 \text{ pfu/cell}\). In most experiments, a control culture of uninfected cells received identical treatment (except for the lack of virus), which served as a dummy infection procedure.
2.3 Plaque Assays

Virus for plaque assays was diluted in phosphate buffered saline, PBS (Dulbecco and Vogt, 1954), containing 1% tryptose phosphate broth. To reduce aggregation, the first dilution was usually in 0.05 M glycine buffer pH 2.5, followed 5 minutes later by 9 volumes of PBS adjusted to pH 8 with 1 N NaOH. Normally 0.1 ml of the dilutions were used for assay in the agar cell-suspension plaque technique (Cooper, 1961).

To make the agar medium, molten 3% Difco Bacto-Agar at 50°C was mixed with an equal volume of a solution containing the following compounds (mg/10ml): NaCl 160, KCl 10, NH₄Cl 1, NaH₂PO₄ 10, MgCl₂·6H₂O 4, NaHCO₃ 4.2, CaCl₂ 2, phenol red 0.1, L-glutamine 2, L-glutamic acid 6, L-arginine 5, inositol 0.2, galactose 30, penicillin 5, streptomycin 2.5, neomycin 2.5. To this was added 5% (by volume) of a 10% suspension of skim milk powder, 5% of a 10% tryptose phosphate broth, 8% calf serum and sufficient 1 N NaOH to bring the pH to 7.3. For screwcapped aspirin bottles and 4" Petri dishes the base layer contained 7.5 and 15 ml respectively of agar medium; the overlay contained 1 and 2 ml. The respective amounts of cell suspension (6 x 10⁶ cells/ml) in the overlay were also 1 and 2 ml.

The aspirin bottles and Petri dishes (in large plastic bags) were incubated for 3 days at 37.2°C and 4 days at 33.5°C or 34.0°C in water baths heated by thermostatically controlled units accurate to ±0.05°C.
(Manufacturers - Buhler, Tubingen; Braun, Melsungen). The cells in the bottles were stained by adding 2 ml/bottle of a solution containing 0.8% NaCl, 7% glucose, 0.017% sodium azide and 0.12% INT stain (i.e. 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride; Cooper, 1959). The cells in the Petri dishes were sprayed from an atomizer with 1.5% INT, 4% glucose in 80% ethanol. After the addition of the stain, both bottles and plates were incubated at 37°C for a further 3 hours.

2.4 Preparation of Cytoplasmic Extracts and their Analysis on Sucrose and Caesium Chloride Gradients

(a) Preparation of cytoplasmic extracts and analysis on sucrose gradients

All operations were carried out at 0-5°C. The cells were chilled, washed twice with PBS and then RSB (0.01 M NaCl, 0.0015 M MgCl$_2$, 0.01 M Tris pH 7.4) was added to give a cell concentration of 2-5 x 10$^7$/ml. After 10 minutes the cells were broken in a glass Dounce homogenizer and the nuclei removed by centrifugation. Sodium deoxycholate and then Brij 58 to a final concentration of 0.5% of each were added to the cytoplasm. This extract was layered directly on a gradient (see below), or in some cases where specifically mentioned, was placed over 1.5 ml of 30% sucrose in RSB and centrifuged 90 minutes at 49,000 rpm in the Spinco type 50 angle rotor. The pellet was then resuspended in the appropriate buffer before layering on a sucrose gradient in the same buffer. As well as RSB, NEB 1 (0.01 M NaCl, 0.01 M EDTA, 0.01
M Tris pH 7.4), HMB (0.01 M NaCl, 0.005 M MgCl₂, 0.01 M Tris pH 7.4) and NEB 2 (0.2 M NaCl, 0.01 M EDTA, 0.01 M Tris pH 7.4) were used as solvents for sucrose gradients.

Sucrose concentrations are expressed as a percentage w/w. The gradients were produced with a gradient maker designed to give linear gradients, and those checked by the measurement of refractive indices of the fractions were indeed linear. Centrifugation was carried out in the Spinco SW 25.1, SW 25.3 or SW 27 rotors. The gradients were fractionated from the bottom by a tube inserted down through the gradients from the top.

The sedimentation constants for native ribosomes and large subunits were taken as approximately 74S and 60S respectively (Girard, Latham, Penman and Darnell, 1965). The sedimentation constant for mammalian native small subunits is generally accepted to be 40 - 45S (Spirin, 1969), and throughout this thesis a value of 45S is used (Girard et al., 1965). The sedimentation constants for the large and small ribosomal subunits in the presence of EDTA were taken as approximately 50S and 30S (Warner and Pène, 1966). The approximate quantities of ribosomes in μg used for fixation (next section) were calculated from the absorbance at 260 nm using a value of 144 for E₁cm

(b) Fixation with glutaraldehyde and caesium chloride gradients

The techniques used were essentially those described by Baltimore and Huang (1968). Aliquots of the
fractions of the sucrose gradient to be fixed were pooled, placed in ice, and the volume made up to 0.8 ml with RSB, HMB, or buffer containing EDTA depending on the particular experiment. Glutaraldehyde, 50% w/w, was diluted with the same buffer to 40% w/v or 8% w/v, allowed to reach room temperature and adjusted to pH 7.0 with 1 M sodium bicarbonate. Within 10 minutes, 0.2 ml of the diluted solution was added to the material to be fixed. The fixed material was then layered within 15 minutes on a preformed CsCl gradient chilled to 0-4°C. Most gradients were 27 - 53% w/w CsCl in RSB containing 0.6% w/w Brij 58, and were formed in a tube of capacity 5.5 ml by pumping 2.0 ml of the 53% and 2.5 ml of the 27% solution from a gradient maker at a rate of 0.3 ml/minute. They were centrifuged at 5°C for 5 hours at 42,000 rpm in the Spinco SW 50.1 rotor and fractionated through a hole punched in the bottom of the tube. The refractive indices at 25°C of alternate fractions were determined with a refractometer (Carl Zeiss, W. Germany), corrected for the contribution of the gradient buffer (0.0012 was subtracted) and the density determined from Tables J-252 to J-255 in the Handbook of Biochemistry published by The Chemical Rubber Co. (1968). The acid-insoluble radioactivity in 100 μl litre of each gradient fraction was determined as in (d) below; approximately 70% of the radioactivity layered on each gradient was recovered in the fractions.
<table>
<thead>
<tr>
<th>Dioxan-Based Scintillation Fluid</th>
<th>Channel Settings Including Gain</th>
<th>% Spillover</th>
<th>Efficiency</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>$^3$H 50-200 50% $^{35}$S 250-1000 9%</td>
<td>Into $^3$H Channel 11 From $^3$H Channel &lt;0.2</td>
<td>$^3$H 9</td>
</tr>
<tr>
<td></td>
<td>$^3$H 50-175 50% $^{14}$C 200-1000 10%</td>
<td>3.6 &lt;0.2</td>
<td>$^3$H 8 $^{14}$C 56</td>
</tr>
<tr>
<td></td>
<td>$^3$H 50-175 50% $^{125}$I 200-1000 10.5%</td>
<td>29 &lt;0.5</td>
<td></td>
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</tbody>
</table>

<table>
<thead>
<tr>
<th>Toluene-Based Scintillation Fluid</th>
<th>Channel Settings Including Gain</th>
<th>% Spillover</th>
<th>Efficiency</th>
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<tr>
<td></td>
<td>$^3$H 50-200 50% $^{35}$S 250-1000 9%</td>
<td>9.8 &lt;0.2</td>
<td>$^3$H 10</td>
</tr>
<tr>
<td></td>
<td>$^3$H 50-175 50% $^{14}$C 200-1000 10%</td>
<td>9.1 1.9</td>
<td>$^3$H 8 $^{14}$C 61</td>
</tr>
</tbody>
</table>

Table 1. Settings on the scintillation counter for the simultaneous counting of two radioactive isotopes.
(c) Determination of the absorbance at 260 nm of sucrose gradient fractions

The $A_{260}$ of each fraction was measured in a 1 cm quartz cell in a Unicam SP 500 Series 2 Spectrophotometer.

(d) Determination of acid-insoluble radioactivity in fractions from gradients

Samples of 10 to 100 μlitre were placed on Whatman 3MM 2.0 cm discs. The discs were dried and washed for five minutes with the following at 0-5°C; three times with 5% trichloroacetic acid (TCA), once with a 1:1 mixture of ether and ethanol, and finally twice with ether. When the discs had dried, they were placed in a vial containing 5 ml of toluene-based scintillation fluid with 0.5% w/v and 0.05% w/v POPOP. The channel settings on the Packard Tri-Carb Liquid Scintillation Spectrometer for the simultaneous counting of two isotopes are given in Table 1. The percentage spillover between channels was calculated each time the counter was used.

2.5 Preparation of Radioactive Virus and Empty Capsids

Cells were infected at a multiplicity of 80 pfu/cell by stirring for one hour on ice with virus suspended in growth medium, and then incubated at 37°C for one hour in Eagle's medium lacking amino acids but containing 200 μg/ml guanidine carbonate. The guanidine was removed by washing the cells twice with cold PBS. The cells were resuspended in medium lacking amino acids (2 x $10^6$ cells/ml) and stirred a further hour at 37°C before [$^3$H] or [$^{14}$C] Reconstituted Protein Hydrolysate was added to 1 μc/ml.
Figure 2 Preparative CsCl gradients of empty capsids and virions. 2.5g of CsCl dissolved in 5 ml of 0.02 M phosphate buffer pH 7.0 was mixed with labelled particles and centrifuged to equilibrium as described in section 2.5. The densities were determined from the refractive indices of the fractions.
Figure 3  Sedimentation through a 15-30% sucrose gradient in NEB2 of naturally occurring empty capsids prepared as described in section 2.5. Centrifugation was for 14.5 hr at 24,000 rpm in the Spinco SW 25.1 rotor. Sedimentation was from right to left.
After 4.5 hours the cells were frozen, thawed and centrifuged 10 minutes at 10,000g. The supernatant was centrifuged for 70 minutes at 200,000g. The pellet obtained was resuspended in 5 ml of 0.02 M phosphate buffer pH 7.0 containing 2.5g of CsCl, and then centrifuged for 20 hours at 48,000 rpm in the Spinco SW 65L titanium rotor. The resulting gradient was fractionated and the peaks of radioactivity corresponding to densities of 1.29 g/cc and 1.34 g/cc were recentrifuged in CsCl solution. Typical gradients are shown in Figure 2.

(a) Virus

The peak fractions from the CsCl gradient at 1.34 g/cc were dialysed against 0.01 M phosphate buffer pH 7.0 at 4°C. Sodium dodecyl sulphate (SDS) and 2-mercaptoethanol were added to 1% and 0.1% respectively and the virus suspension heated at 100°C for one minute. After dialysis against 0.01 M phosphate buffer pH 7.3, 0.1% SDS and 0.1% 2-mercaptoethanol, the preparation was ready for electrophoresis.

(b) Top component or naturally occurring empty capsids

The pooled fractions from the CsCl gradient at 1.29 g/cc were dialysed against NEB 2 buffer and centrifuged through a 15-30% w/w linear sucrose gradient in NEB 2 buffer (Figure 3). The peak fractions at approximately 70S were prepared for electrophoresis using SDS and 2-mercaptoethanol as described above for the virus.
Figure 4  Sedimentation through a 5-30% sucrose gradient in RSB of artificial empty capsids prepared as described in section 2.5. Centrifugation was for 5 hr at 24,000 rpm in the Spinco SW 25.1 rotor.
(c) **Production of artificial empty capsids**

The method used was similar to that of Van Elsen and Boeyé (1966). Virus from the CsCl gradient was first dialysed against 0.01 M phosphate pH 7.0, then against 0.1 M borate buffer pH 10.5, and finally heated at 40°C for 35 minutes. The disrupted virions were dialysed against cold RSB and NEB buffer. The empty capsids moved as a single peak at approximately 70S through a 5-30% w/w linear sucrose gradient in RSB (Figure 4).

2.6 **Polyacrylamide Gel Electrophoresis**

(a) **General procedure**

The methods used for the electrophoresis of proteins in polyacrylamide gels containing sodium dodecyl sulphate were essentially those described by Burrell, Martin and Cooper (1970). The gels contained 0.1% SDS, 0.5 M urea, 0.1 M phosphate buffer pH 7.3, 7.5% or 10% (w/v) acrylamide and N, N'-methylenebisacrylamide at 2.7% of the acrylamide concentration. 7.5% gels were used for the work described in Chapter Three, and 10% gels for that in Chapter Four. The acrylamide/bisacrylamide solution was de-aerated and the total gel solution polymerised at room temperature with 0.012% (v/v) N, N, N', N'-tetramethylenediamine and 0.075% (w/v) ammonium persulphate in glass tubes with an internal diameter of 6 mm. The tubes were polished internally beforehand with "Pledge", a commercial brand of silicone furniture polish, to facilitate extrusion. The electrophoresis buffer was 0.1% (w/v) SDS,
0.01% (v/v) thioglycollic acid in 0.1 M sodium phosphate buffer pH 7.3. Before use, each gel was electrophoresed for 1-2 hours with 0.1 ml each of 0.1 M reduced glutathione and dithiothreitol (100 mg/ml).

Ribosomes or subunits in sucrose gradients were prepared for electrophoresis by centrifuging the appropriate fractions at 49,000 rpm for 5 hours in the Spinco type 50 rotor and resuspending the pellet in 0.01 M sodium phosphate pH 7.3, 2% SDS, 1% 2-mercaptoethanol, 0.5 M urea (SUMP). Alternatively, acid-insoluble material was precipitated from the fractions of sucrose gradients with cold 10% TCA and washed with 5% TCA and acetone before treatment with SUMP. In both cases the SUMP buffer was heated at 100°C for 90 seconds. In some cases the preparations were then dialysed overnight against 0.01 M phosphate buffer pH 7.3, 0.1% SDS and 0.1% 2-mercaptoethanol, but this step had no apparent effect on the final migration of proteins and was usually omitted. The samples, not exceeding 0.2 ml, were layered on 15 or 20 cm gels. One drop of 30% sucrose and 1% bromophenol blue or phenol red solution was added, electrophoresis buffer layered on top, and the preparations electrophoresed at 25°C for 12-18 hours at a current of 6-8 mA/gel. After electrophoresis the gels were extruded from the tubes, frozen and cut into 1.6 mm slices, each slice placed in a vial, 0.5 ml of 10% (v/v) piperididine added, the slices dried overnight at 60°C, 0.5 ml of water added and followed two hours later by 10 ml of
dioxan-based scintillation fluid (see section 2.7). The vials were chilled to 5°C and stood 2 hours before counting. The channel settings are given in Table 1.

(b) **Staining**

Some gels were not sliced but stained overnight with 0.5% Coomassie Brilliant Blue in a solvent of 5 parts methanol, 5 parts water and 1 part glacial acetic acid. Several changes of the same solvent were used to progressively destain the gels.

(c) **Preparation of 125I-labelled proteins**

The majority of the labelled standard proteins were prepared by Dr P.D. Cooper using the method of Greenwood, Hunter and Glover (1963). Two milligrams of protein were dissolved at room temperature in 100 μlitre of 0.01 M sodium phosphate at pH 7.3. Ten microcuries of carrier-free [125I]-NaI was added, followed by 5 μlitre of a 10 mg/ml solution of chloramine T (sodium salt of N-chloro-p-toluenesulphonamide). After 3 minutes the reaction was stopped by adding 50 μlitre of a 24 mg/ml solution of sodium metabisulphite and then 50 μlitre of 1 mM potassium iodide solution. Unreacted [125I]-iodide was removed by dialysis or passage through a small (1 ml) Sephadex column. A large excess of protein was used to reduce the ratio of [125I] atom/protein molecule to an insignificant level (<10⁻³), so that no protein molecule received more than one iodine atom. The labelled protein did not migrate differently upon electrophoresis from the unlabelled protein, as judged by staining and counting the same gel.
The molecular weights in daltons used for the iodinated proteins were as follows: human serum albumin (HSA) 70,000 and *Jasus lalandii* (the Southern Crayfish) hemocyanin 86,000 (Parish and Marchalonis, 1970), trypsin 23,800 (Kay, Smillie and Hilderman, 1961; Keil, 1971), pepsin 33,600, and pepsinogen 39,000 (Fruton, 1971), tobacco mosaic virus protein 17,650 (from the amino acid sequence in "Atlas of protein sequence and structure", vol. 5), bovine cytochrome c 12,500 (Okunuki, 1966). The molecular weights assigned to other proteins after electrophoresis were calculated from a plot of the logarithm of the molecular weight against the distance migrated (Shapiro, Viñuela and Maizel, 1967), using the above proteins as standards.

2.7 Measurement of the Incorporation by Cells of Labelled Amino Acids into Acid-Insoluble Material

Samples of 10^6 cells were taken from a cell suspension in radioactive medium and immediately added to an equal volume of PBS standing in ice. The cells were sedimented by centrifugation, washed once with PBS, resuspended in 2 ml of 1N NaOH, and stood at room temperature for 20 minutes. After the addition of 500 μg of bovine plasma albumin as carrier, 8 ml of cold 10% trichloroacetic acid (TCA) were added and the samples placed on ice for 10 minutes. The precipitates were collected by centrifugation, washed twice with cold 5% TCA and then resuspended in 0.5 ml of 5% TCA, followed by
10 ml of dioxan-based scintillation fluid containing 15\% (w/v) naphthalene, 0.23\% (w/v) PPO and 0.009\% (w/v) POPOP.

2.8 Preparation of Nuclei and Aggregate Enzyme for Assay of DNA-Dependent RNA Polymerase Activity

(a) Preparation of nuclei

Cells were chilled and washed twice with cold PBS (Dulbecco and Vogt, 1954). Next RSB (0.01 M NaCl, 0.0015 M MgCl$_2$, 0.01 M Tris pH 7.4) containing 5 mM 2-mercaptoethanol was added to give a cell concentration of $5 \times 10^7$/ml. Every procedure was done at 0-5°C. After 10 minutes the cells were broken in a glass Dounce homogenizer, and the suspension adjusted to 0.25 M sucrose, 1 mM MgCl$_2$, 5 mM 2-mercaptoethanol by adding 1.25 M sucrose, 5 mM MgCl$_2$, 25 mM 2-mercaptoethanol (5 x SMM). The nuclei were separated by centrifugation, washed twice with SMM, and resuspended in 0.25 M sucrose, 5 mM MgCl$_2$, 10 mM potassium phosphate buffer pH 6.5. Glycerol (95\%) containing 5 mM MgCl$_2$ was added to give 70\% glycerol and the suspension stored at -60°C in 2 ml lots containing 0.2 ml of packed nuclei. Nuclei kept this way retain RNA polymerase activity for at least 2 months (Read and Mauritzen, 1970). Before assay the suspension was thawed, the nuclei washed twice with SMM and finally resuspended in SMM to the original storage volume.

(b) Preparation of aggregate enzyme

Not less than $4 \times 10^8$ cells were washed, suspended in 0.1% Tween 80 (Fisher and Harris, 1962) at $5 \times 10^7$
cells/ml and gently disrupted in a Dounce homogenizer. The nuclei were washed three times by centrifugation through sucrose as above. The final nuclei pellet was resuspended in seven volumes of 0.05 M Tris buffer pH 7.4 containing 5 mM 2-mercaptoethanol, allowed to stand for 10 minutes and ultrasonicated for 5-10 seconds to lyse the nuclei. 2 M KCl was added to a final concentration of 0.4 M as described by Weiss (1968). The aggregate was wound out on a rod and washed twice by gentle stirring in cold KCl-Tris solution. After draining, the gel was homogenized in 0.05 M Tris buffer pH 8.0, and used for assay or electrophoresis.

Before electrophoresis, deoxyribonuclease 1 was added to 25 μg/ml and the mixture incubated at 37°C until cleared. SDS and 2-mercaptoethanol were added to 2% and 1% respectively. After 3 minutes at 100°C, the preparation was dialysed overnight in 0.01 M sodium phosphate buffer pH 7.3, 0.1% SDS and 0.1% 2-mercaptoethanol.

(c) DNA-dependent RNA polymerase assays

Enzyme activity was followed by measuring the incorporation at 37°C of [3H] GTP or [3H] CTP (lc/m mole) into acid-insoluble material. The components of the assay mixtures are given in the relevant tables of section 4.2. Each assay was done in duplicate. The reaction was stopped by placing the assay tube in ice, adding 0.5 ml of 0.1 M sodium pyrophosphate, 0.1 ml of 10 mg/ml bovine plasma albumin and 6 ml of 0.5 M perchloric acid. The acid-insoluble precipitate was collected by centrifugation,
washed three times with 5 ml of 0.5 M perchloric acid, once with ethanol/ether (1:1) and dried.

Water (0.1 ml) and 0.2 ml of Hydroxide of Hyamine 10x were then added. After 1 hour at 60°C the precipitate had dissolved. The sample was mixed with 10 ml of toluene scintillation fluid containing 20% (v/v) absolute ethanol, 0.5% (w/v) PPO and 0.05% (w/v) POPOP.

Protein was determined by the method of Lowry et al. (1951) using bovine plasma albumin as a standard.

2.9 Source of Chemicals

α-amanitin;

Acrylamide and N,N'-methylenebis-acrylamide;

Actinomycin D;

Auto-Pow;

Bovine cytochrome c;

Bovine pancreatic ribonuclease;

Brij 58;

Caesium chloride;

Gift from Dr T. Wieland, Max Planck Institute for Medical Research.


Merck, Sharp and Dohme, West Point.

Flow Laboratories Inc., Scotland.

Sigma Chemical Co., Missouri.

Worthington Biochemical Corp., New Jersey.

Atlas Chemical Industries, Wilmington.

Ajax Chemicals Ltd., Sydney - Melbourne.
Deoxyribonuclease I;

1,4-Dioxan and Toluene
   (Analytical Reagent);
Glutaraldehyde solution,
   50% w/w;
Guanidino carbonate;
Guanidine hydrochloride;
Hemocyanin;

[3H]GTP and [3H]CTP,
   lc/m mole;
Human serum albumin;

Iodine - 125, Code IMS 3;

L - leucine - 4,5 - T,
   lc/m mole;
L - lysine (4,5 - 3H),
   50c/m mole;
L - methionine - S^{35},
   50c/m mole;
Naphthalene;
Pepsin;
Pepsinogen;

Worthington Biochemical Corp.,
   New Jersey.
Ajax Chemicals Ltd., Sydney -
   Melbourne.
Fisher Scientific Company, New
   Jersey.
Fluka, Switzerland.

Provided by Mr R.E. Langman,
   Dept. of Microbiology,
   J.C.S.M.R.

Schwarz/Mann, New York.

Nutritional Biochemicals Corp.,
   Ohio.
The Radiochemical Centre,
   Amersham.
The Radiochemical Centre,
   Amersham.
Schwarz/Mann, New York.
The Radiochemical Centre,
   Amersham.
Matheson, Coleman and Bell, Ohio.
Worthington Biochemical Corp.,
   New Jersey.
Worthington Biochemical Corp.,
   New Jersey.
Piperidine; Fluka, Switzerland.
Pledge; Johnson & Son, N.S.W.
POPOP, i.e. 1,4 - bis - (5-phenyloxazole) - benzene;
POPOP, i.e. 2,5 - Diphenyl- oxazole; Koch - Light Laboratories Ltd., England.
Puromycin dihydrochloride; Nutritional Biochemicals Corp., Ohio.
Reconstituted Protein Hydrolysate, \(^3\)H- or \(^14\)C-labelled;
Sheep IgG - purified; A gift of Mr P. Ey, Dept. of Microbiology, J.C.S.M.R.
Skim Milk; Dutch Jug, (commercial brand).
Sodium dodecyl sulphate; Matheson, Coleman and Bell, Ohio.
Sucrose - ultra pure;
Tobacco Mosaic Virus Protein;
Trypsin; Worthington Biochemical Corp., New Jersey.
Tween 80; Selbys, N.S.W.
27 c/m mole;
CHAPTER THREE

VIRAL PROTEINS IN RIBONUCLEOPROTEIN COMPLEXES OF POLIOVIRUS-INFECTED CELLS

3.1 Introduction

3.2 The Sedimentation Coefficient of Empty Capsids and the Molecular Weights of Empty Capsid and Virion Proteins

(a) Sedimentation coefficient of empty capsids
(b) Molecular weights of VPO, VP1, VP2 and VP3

3.3 Viral Proteins in Ribonucleoprotein Complexes in Infected Cells of Sedimentation Coefficient between 20S and 80S

(a) Viral proteins attached to native 45S ribosomal subunits of infected cells
(b) Viral proteins attached to ribonucleoprotein complexes of 60 - 80S in infected cells
(i) Cross-fixing with glutaraldehyde
(ii) Evidence for ribosomal complexes containing viral protein
(c) The effect of guanidine on ribonucleoprotein complexes carrying viral protein
(d) Ribonucleoprotein complexes in infected cells labelled with [³H] uridine
(e) Attempts to dislodge VPO, VP1 and VP3 from the 60 - 80S ribonucleoprotein complexes

3.4 Viral Proteins in the Guanidon and the Replication Complex
(a) Labelling with $[^3\text{H}]$uridine: confirmation of the guanidon

(b) Labelling with $[^3\text{S}]$methionine and the probable presence of VP0, VP1 and VP3 in the replication complex and guanidon

(c) Release of VP0, VP1 and VP3 from the replication complex

3.5 The Inhibition of Ribosomal Protein Synthesis by Infection with Poliovirus

(a) Inhibition of total protein synthesis

(b) Inhibition of ribosomal protein synthesis

3.6 Discussion

(a) The small ribosomal subunit

(b) Complexes of viral ribonucleoprotein and ribosomes

(c) The effect of guanidine on ribosomal complexes

(d) The replication complex and guanidon

(e) The polypeptide composition of empty capsids and virions

3.7 Summary
3.1 Introduction

The major part of this chapter presents evidence for the existence of the viral structural proteins VP0, VP1 and VP3 in ribonucleoprotein complexes within infected cells. It was first necessary to resolve contradictions in the literature that hindered identification of these proteins (section 3.2). For the reasons discussed in Chapter One, section 1.5, native small ribosomal subunits were examined following infection for the presence of new proteins that might explain the inhibition of host protein synthesis and the initiation of protein synthesis on viral RNA as messenger. Having found VP0, VP1 VP3 apparently attached to the small subunit [section 3.3(a)], the search for viral proteins was extended to other ribosomal complexes [sections 3.3(b),(c),(d)] and the replication complex (section 3.4), using a combination of sucrose and caesium chloride gradient centrifugation, and gel electrophoresis, to identify labelled ribonucleoprotein species. A significant artifact introduced by cross-fixing with glutaraldehyde was discovered, and the reasons for its presence partially resolved. Experiments on the inhibition of ribosomal protein synthesis after infection are also described (section 3.5).

The possible significance for picornavirus growth of the findings in this chapter is discussed in Chapter Six in terms of the equestron model (Cooper et al., 1973). The more direct interpretations of the results are discussed in section 3.6.
3.2 The Sedimentation Coefficient of Empty Capsids and the Molecular Weights of Empty Capsid and Virion Proteins

When cells were labelled with radioactive amino acids later than two hours after infection in the presence of guanidine, top component or naturally occurring empty capsids were detected. This added to the difficulty of studying ribosomal protein because empty capsids are generally accepted to have a sedimentation constant of 73-80S, almost the same as native ribosomes (Maizel et al., 1967; Jacobson and Baltimore, 1968b), although Penman, Becker and Darnell (1964) first reported a value of about 65S. The available estimates for the molecular weights in daltons of the polypeptides constituting the empty capsids of the Mahoney strain, i.e. Vp0, Vp1, Vp3 were 41,000, 35,000, 24,000 (Maizel and Summers, 1968) and 40,000, 35,000, 23,000 (Jacobson et al., 1970) respectively. Throughout this thesis the term Vp0 (Jacobson and Baltimore, 1968b) rather than NCVP6 (Maizel et al., 1967) will be used to denote the largest polypeptide in empty capsids.

Initial experiments yielded components that apparently were empty capsids, but in the particular sucrose gradients used had a sedimentation constant relative to ribosomes different from the generally accepted value of 73-80S. The molecular weights of the constituent polypeptides of these components were also different from those given above for Vp0, Vp1 and Vp3. This section therefore describes a more accurate characterization of the
Figure 5  Cytoplasmic extracts of infected and uninfected cells were prepared and treated as described in the text before being centrifuged through a 15-30% sucrose gradient in NEB2 for 16 hr at 24,000 rpm in the Spinco SW 25.1 rotor.
properties of empty capsids and presents data relevant to
the studies on ribosomal-associated proteins in the
following pages.

(a) Sedimentation coefficient of empty capsids

The $S$ values of Mahoney and $ts^+$ empty capsids were
indistinguishable. The following illustrative experiment
involves $ts^+$. $4 \times 10^7$ cells were infected with the $ts^+$
strain of virus and incubated for 2 hours in Eagle's
medium containing actinomycin D (0.3 $\mu g/ml$) and guanidine
carbonate (200 $\mu g/ml$). The cells were then washed free of
the drugs with PBS and incubated a further 2.5 hours in
Eagle's medium lacking leucine, before $[^3H]$leucine to a
concentration of $6 \mu c/ml$ was added. After one hour's
incubation, a cytoplasmic extract was prepared and
centrifuged through a sucrose cushion as described in
section 2.4(a). The pellet was resuspended in NEB2 buffer
and analysed on a sucrose gradient (Figure 5B). $4 \times 10^7$
uninfected cells were treated in the same way except for
the use of a dummy inoculum in the infection procedure
(Figure 5A). The peaks of radioactivity in the gradient of
uninfected cell extract coincided with the absorbance peaks.
The main peak of radioactivity in the gradients of infected
cell extracts from several experiments was always at
60-65S (e.g. fractions 11, 12 of Figure 5B), calculated
using values of 50S and 30S for the subunits (Warner and
Pène, 1966), and differed in the molecular weights of its
constituent polypeptides from those previously reported
for empty capsids (see later). Thus it was necessary to
Figure 6  Fractions 11,12,13 of Figure 5B were pooled. One portion was treated with ribonuclease (B) and an equal amount (A) mixed with $^{14}$C-labelled artificial empty capsids [prepared as in section 2.5(c)] before centrifugation through a 15-30% sucrose gradient in NEB2 at 24,000 rpm for 16 hr in the Spinco SW 25.1 rotor.
determine whether this peak represented empty capsids or labelled protein attached to a small proportion of the large ribosomal subunits. The following experiments established beyond doubt that the main peak of radioactivity comprised empty capsids.

Material in fractions 11, 12, 13 of Figure 5B was pooled. One portion was treated with 200 μg or 8 units of bovine pancreatic ribonuclease for 30 minutes at 37°C. An equal amount was mixed with artificially produced empty capsids prepared as described in section 2.5(c) and labelled with [14C]amino acids. Both samples were centrifuged on 15-30% sucrose gradients in NEB2. From Figure 6B it can be seen that the ribonuclease had no effect on the 3H-labelled material, which almost cosedimented with artificially produced empty capsids (Figure 6A). The lack of label at the top of the gradient in Figure 6B indicated that no radioactivity could be regarded as bound to the 50S ribosomal subunit.

Thus the naturally occurring empty capsids of the ts+ strain of virus were shown to have a sedimentation constant in these gradients of about 65S relative to the ribosomal subunits. The empty capsids of the Mahoney strain were also subsequently found to have this S value relative to ribosomes and subunits in the gradients shown later in Figures 11B, 11C and 28 of section 3.3. In addition the S value of empty capsids was calculated from Figures 5B, 11B, 11C and 28 using the method of McEwen (1967), which enables the $s_{20,w}$ value of a particle (i.e. the S value in a solvent
Figure 7 (A) Co-electrophoresis through 7.5% gel of ts+ empty capsids (fractions 11-13, Figure 5B) with ¹²⁵I-labelled standard proteins. The empty capsids were prepared for electrophoresis by precipitation with TCA as described in section 2.6(a). (B) The molecular weight of each standard protein plotted on a logarithmic scale against the distance migrated through the gel in (A).
with the density and viscosity of water at 20°C) to be estimated from linear sucrose gradients. For these calculations the density of empty capsids was taken as 1.30 g/cc. The mean value and standard deviation of the four determinations for $s_{20,w}$ was 77S ± 2S. However, for practical purposes throughout the remainder of this thesis, the sedimentation constant of empty capsids will be referred to as 60-65S i.e. the apparent value in linear sucrose gradients relative to ribosomes (Girard et al., 1965; Warner and Pène, 1966).

(b) **Molecular weights of VP0, VP1, VP2 and VP3**

More accurate determinations of the molecular weights of VP0, VP1, VP2 and VP3 than those of Jacobson et al., (1970) and of Maizel and Summers (1968) were possible because of the use of radioactively-labelled standard proteins of known characteristics. The position of a band of labelled standard protein after electrophoresis in a polyacrylamide gel containing sodium dodecyl sulphate can be determined more precisely in relation to labelled unknown proteins if the gel is sliced and counted than if it is stained and examined visually. Thus $^{125}$I-labelled standard proteins were co-electrophoresed with $^{3}$H-labelled viral proteins and the molecular weights of the latter estimated by the method of Shapiro et al. (1967).

The three main polypeptides present in the 60-65S peak (fractions 11-13) of Figure 5B are shown in Figure 7A, and the curve used to calculate the molecular weights of VP0, VP1 and VP3 shown in Figure 7B. A precisely linear
Figure 8  Co-electrophoresis of $^{14}$C-labelled $ts^+$ virions [section 2.5(a)] with naturally occurring $^3$H-labelled $ts^+$ empty capsids isolated (A) from a CsCl gradient [section 2.5(b)] and (B), from a sucrose gradient (as for Figures 5 and 7).
Figure 9  (A) Empty capsids of the Mahoney strain of virus (fractions 10-12 of Figure 11B) precipitated with TCA as in section 2.6(a) were co-electrophoresed with $^{125}$I-labelled standard proteins (7.5% gel).  (B) The molecular weight of each standard protein plotted on a logarithmic scale against the distance migrated through the gel in (A).
relationship between the distances migrated and the logarithms of the molecular weights of the standard proteins was not maintained over the range of molecular weights used. The mean molecular weights and standard deviations in daltons from three determinations of VPO, VP1 and VP3 of ts were 34,100±500, 28,300±70 and 22,000±400. The close similarity in gel pattern of top component (Maizel et al., 1967) or empty capsids prepared from a caesium chloride gradient [section 2.5(b)] to the material obtained in an experiment like the one described above can be seen in Figure 8, in which 14C-labelled ts+ virions were co-electrophoresed with 3H-labelled ts+ empty capsids from the two sources. A minor polypeptide of molecular weight 25,000 was also present.

The molecular weights of VPO, VP1 and VP3 in Mahoney empty capsids were determined in five separate experiments. One such experiment is shown in Figure 9. Again there was a minor polypeptide of 25,000 daltons in the empty capsids, and the semi-logarithmic plot for the standard proteins was not a straight line. However, in both Figures 7B and 9B, the deviation from a linear relationship was evident only if the standard protein larger than pepsin was included in the graph. The means and standard deviations in daltons of the five determinations of the molecular weights of VPO, VP1 and VP3 from Mahoney empty capsids were 34,100±700, 30,200±400 and 22,300±400 respectively.
Figure 10. (A) Co-electrophoresis of the polypeptides of Mahoney (\(^{35}\)S-labelled) and ts\(^+\) (\(^{3}\)H-labelled) empty capsids. (B) Co-electrophoresis of the polypeptides of Mahoney empty capsids (\(^{35}\)S-labelled) and virions (\(^{3}\)H-labelled). (C) Co-electrophoresis of Mahoney empty capsids (\(^{3}\)H-labelled) and \(^{125}\)I-labelled pepsinogen. The \(^{3}\)H-labelled material was prepared as described in section 2.5, except that the ts\(^+\) empty capsids were labelled with \(^{3}\)Hleucine, not RPH. The \(^{35}\)S-labelled material was prepared as for Figure 29 (see later).
The similarities in the molecular weights of ts+ and Mahoney VP0 and VP3, together with the differences in the molecular weights of VP1 (Cooper, Summers and Maizel, 1970) were further demonstrated by co-electrophoresing the empty capsids of the two strains (Figure 10A). The molecular weight of VP2 from ts+ was 26,500 daltons calculated from Figure 8. The molecular weight of Mahoney VP2 calculated from Figure 10B was 27,000 daltons. Hence VP2 of the two strains was of similar size.

Finally, the observation that VP0 had a molecular weight substantially less than 39,000 daltons was confirmed by using 125I-labelled pepsinogen as a molecular weight marker (Figure 10C). Clearly VP0 migrated more rapidly than pepsinogen, which has a molecular weight of 39,000 daltons.

3.3 Viral Proteins in Ribonucleoprotein Complexes in Infected Cells of Sedimentation Coefficient between 20S and 80S

Newly synthesized proteins on the native ribosomes and subunits were examined by labelling infected cells with radioactive amino acids during the period from 3 to 5 hours after infection. The general procedure was as follows. Cells were infected at 0°C for 2 hours with the Mahoney strain of virus at a multiplicity of 20-30 pfu/cell and incubated at 37°C in Auto-Pow medium for 3 hours at 10^6 cells/ml. The culture was then divided into two equal portions, centrifuged and resuspended in Eagle's medium lacking all amino acids, at 4 x 10^6 cells/ml.
Figure 11 8 x 10^7 cells were infected with Mahoney virus, incubated 3 hr at 37°C in Auto-Pow medium, resuspended in Eagle's medium lacking amino acids but with [3H]RPH at 5 μc/ml, and incubated a further 2 hr. Half the cells were labelled in the presence of 2 mM guanidine hydrochloride. 5 x 10^7 uninfected cells were treated in the same way except for the absence of virus and guanidine, and the use of [14C]RPH. Cytoplasmic extracts were centrifuged through 15-30% sucrose gradients in RSB for 16 hr at 22,000 rpm in the Spinco SW 25.1 rotor. Sedimentation was from right to left. (A) Uninfected cells. (B) Infected cells (no guanidine). (C) Infected cells treated with guanidine.
Guanidine hydrochloride to a final concentration of 2 mM was added to one culture. Five minutes later, \(^{3}H\)-labelled Reconstituted Protein Hydrolysate (RPH) was added to both cultures to give a concentration of 5 \(\mu\)c/ml. Uninfected cells were treated in a similar way except for the use of virus-free medium in the "infection" procedure and the use of \(^{14}C\)RPH as label at a concentration of 1.8 \(\mu\)c/ml. After a further 2 hours at 37°C, the cultures were chilled and washed, cytoplasmic extracts were prepared, layered on sucrose gradients in RSB and centrifuged in the Spinco SW 25.1 rotor (Figure 11). As expected, in uninfected cell extracts the peaks of radioactivity coincided with the peaks of absorbance at 74S, 60S, and 45S, i.e. fractions 8, 12 and 18 respectively in Figure 11A. In infected cells, the greater part of the labelled protein was expected to be virus-specified (Summers et al., 1965), with the most noticeable peak at 65S (empty capsids). The significant features of the gradients of infected cell extracts that will be discussed in this section were as follows:

1. the peak of radioactive viral protein at 45S in infected cells (no guanidine),
2. the existence of labelled ribonucleoprotein complexes carrying viral protein in the 60-80S region,
3. the effect of guanidine on the virus-induced labelling of ribosomal and other ribonucleoprotein structures.
Figure 12  The fractions constituting the 45S subunits of 1.2 x 10^8 infected cells from an experiment identical to that in Figure 11B were pooled and centrifuged through a 30-45% sucrose gradient in RSB for 17 hr at 26,000 rpm in the Spinco SW 27 rotor.
The fact that empty capsids have an S value of approximately 65S (fractions 11-13 of Figures 11B, 11C) relative to the ribosomes and subunits was established in the previous section.

(a) **Viral proteins attached to native 45S ribosomal subunits of infected cells**

In view of their likely importance in the initiation of translation of viral protein and in the repression of host protein synthesis by virus, the native 45S subunits were first examined for the presence of attached viral proteins. For further purification, the fractions constituting the 45S subunits in gradients similar to those in Figure 11, e.g. numbers 17-19 Figure 11A, 11B, were pooled and layered directly on 30-45% sucrose gradients which were then centrifuged in the Spinco SW 27 rotor. Figure 12 shows one such second gradient, in this case for the 45S subunits from 1.2 x 10^8 infected cells. There was a large peak of radioactivity corresponding exactly with the 45S subunit; empty capsids were expected to occur at fraction 20, and would contribute negligible radioactivity to this peak. The faster moving peak at fractions 8-11 in Figure 12 corresponded in sedimentation coefficient and polypeptide composition (see later in Figure 15B) to that of pure Mahoney virions, and represented about 0.1% of the total production of virus. A large amount of virus was sedimented through the first gradient, which was
Figure 13 - The labelled proteins in the 45S region, fractions 23-26 of Figure 12, were precipitated with TCA [section 2.6(a)] and co-electrophoresed with $^{125}$I-labelled standard proteins.
Figure 14  The proteins of the 45S subunits of uninfected cells (labelled as for Figure 11A but with $[^3]H$leucine) were precipitated with TCA as in section 2.6(a) and co-electrophoresed with $^{125}I$-labelled standard proteins. The positions of the peaks of the standard proteins are indicated by vertical lines.
Figure 15 Three gels run concurrently. (A) Mahoney naturally occurring empty capsids. (B) Virus from fractions 8-10 in Figure 12. (C) Fractions 21-24 in Figure 11B. Each sample was prepared for electrophoresis by precipitation with TCA and resuspension in SUMP [section 2.6(a)].
fractionated from the bottom, and it appears that a small proportion of virus was washed into the gradient fractions.

The labelled proteins in the 45S region (fractions 23-25) of Figure 12 were analysed by gel electrophoresis (Figure 13). The three major peaks at fractions 33, 37 and 45 corresponded in molecular weight to the three polypeptides found in the naturally occurring empty capsids of the Mahoney strain [34,100, 30,200 and 22,300 daltons; section 3.2(b)]. This result was also obtained in three other experiments in which the 45S subunits of infected cells were prepared as in Figures 11B and 12. The gel pattern of a tritiated preparation of 45S subunits from uninfected cells, with the position of the 125I-labelled standard proteins marked, is shown in Figure 14. None of the peaks corresponded in molecular weight to VP0, VP1 and VP3 in either this gel or a second gel (not shown) in which proteins of empty capsids and 45S subunits of uninfected cells were co-electrophoresed.

The three gels of Figure 15 were electrophoresed concurrently under identical conditions. Figure 15A shows the pattern from Mahoney empty capsids prepared as for Figure 9A, and Figure 15B is the gel pattern of the faster sedimenting peak shown in Figure 12. This peak corresponded in polypeptide composition to that of pure Mahoney virions. The significance of Figure 15C is as follows. The infected cell 45S material for electrophoresis (Figures 12,13) was obtained from a gradient identical to that shown in Figure 11B. To see whether its gel pattern
Figure 16  Isopycnic centrifugation in CsCl after fixation with 8% glutaraldehyde of a portion of the same 45S preparation analysed by gel electrophoresis in Figure 13.
was a result of contamination with material higher in the gradient, fractions 21 to 24 of the gradient of Figure 11B were pooled and electrophoresed (Figure 15C). In these fractions there was more NCVP4 and VP2 than in the 45S preparations (e.g. Figure 13), indicating that the 45S gel pattern did not simply result from contamination by the large amount of radioactivity at the top of the gradient.

A portion of the same preparation of 45S material used to obtain Figure 13 was fixed with glutaraldehyde and the density in caesium chloride determined (Figure 16). Seventy per cent of the recovered radioactivity banded at a density of 1.48 g/cc and 30% at the density of protein. The density of the 45S ribosomal subunits of uninfected cells isolated in RSB was independently determined to be 1.48 g/cc. The presence of radioactivity at the density of protein indicated either incomplete fixation or contamination of the 45S preparation by labelled protein. If the latter was the case, then the level of contamination was insufficient to account for the proportion of radioactivity recovered in the VP0, VP1 and VP3 region (45%) of Figure 13 but explained the small amounts of other polypeptides throughout the gel.

The next experiment was done in collaboration with Dr P.D. Cooper. Another portion of the same 45S material was treated in the following way to disrupt the subunits. Ribonuclease was added to 10 μg/ml, the preparation incubated for 10 minutes at room temperature, and then EDTA, LiCl and sodium deoxycholate added to final concentrations of 20 mM, 2.0 M and 0.2 mg/ml, respectively.
Figure 17  Separate 10-45% sucrose gradients of sheep IgG, human serum albumin (HSA), and 45S subunits from infected cells treated with ribonuclease, EDTA, LiCl and sodium deoxycholate as described in the text. Centrifugation was for 16 hr at 45,000 rpm at 10°C in the Spinco SW 50.1 rotor.
The sample was diluted with 4 volumes of water and centrifuged through a 10-45% sucrose gradient in RSB. In Figure 17, the sedimentation of the label from the disrupted 45S subunits is compared with that of sheep IgG (approximately 7S) and human serum albumin (approximately 5S) in identical gradients; fraction 1 comprised a pad of 60% sucrose. The majority of the label after disruption sedimented at approximately 6S. The radioactivity recovered in the gradient was about 50% of that in the sample treated. The same result was obtained in two further gradients of disrupted 45S subunits from infected cells.

In order to test the possibility that the labelled proteins had fortuitously attached to the 45S subunits after homogenization of the cells and before centrifugation, uninfected cell cytoplasm was mixed with the labelled cytoplasm of an equal number of infected cells (treated as for Figure 11B) from which the ribosomal material had been removed by centrifugation. The mixture was sedimented through a sucrose gradient as in Figure 11 but no label was found associated with the ribosomes or subunits. This suggests that the presence of VP0, VP1 and VP3 on the small subunit had some functional significance in intact infected cells.

A second type of experiment confirmed the polypeptide composition of the 45S particles present in infected cells. The procedure was similar to that described in Figure 11, except that actinomycin D was present during the incubation following infection, labelling
Figure 18  (A) 8 x 10⁷ cells were infected with Mahoney virus, incubated 2.5 hr in Auto-Pow medium containing 0.2 µg/ml of actinomycin D, resuspended in Eagle's medium lacking amino acids but containing [³⁵S]methionine at 15 µc/ml and incubated 1.5 hr. The cytoplasmic extract was centrifuged through a 15-30% sucrose gradient in HMB for 15 hr at 21,000 rpm in the Spinco SW 27 rotor. Equal portions of the 45S region of (A) (fractions 15-17 pooled) were centrifuged through 15-30% sucrose gradients for 15 hr at 22,000 rpm in the Spinco SW 27 rotor, using (B) sucrose dissolved in HMB, or (C) sucrose dissolved in NEBl.
Figure 19 Fractions 13, 14, 15 of Figure 18B were pooled and the TCA insoluble material co-electrophoresed with \(^3\text{H}\)-labelled Mahoney virions prepared as in section 2.5(a).
was commenced 30 minutes earlier, and the cytoplasmic extract was prepared and analysed in a buffer (HMB) differing from RSB only in the MgCl$_2$ concentration, which was 5 mM. This concentration is usually optimal for in vitro mammalian protein synthesis and the binding of initiation factors to the small ribosomal subunit (Shafritz and Anderson, 1970). Figure 18A shows the first sucrose gradient of the cytoplasmic extract. Fractions 15, 16, 17 were pooled and equal portions further analysed on sucrose gradients (Figures 18B, 18C). Approximately 40% of the radioactivity sedimented at 45S between fractions 11 and 18 with HMB as the gradient buffer (Figure 18B), but was shifted to the top of the gradient in the presence of EDTA (Figure 18C). Empty capsids (fractions 7-11 in Figures 18B, 18C) were unaffected by the treatment with EDTA.

The gel pattern for the 45S region in Figure 18B (fractions 13, 14 and 15) is shown in Figure 19. Labelled Mahoney virion protein was co-electrophoresed with the preparation. Once again the three major labelled polypeptides on the subunit were VP0, VP1 and VP3. Other experiments (discussed in section 3.5) have shown that the synthesis of the polypeptide at fraction 10 is probably host-coded, and is more refractory to inhibition after infection than is the synthesis of other polypeptides of the small ribosomal subunit.

To summarize, this section showed that viral proteins were found in particles having the same S value and density as native 45S ribosomal subunits. The proteins
Table 2. The buoyant densities of ribosomes and subunits in CsCl after fixation with glutaraldehyde. Each figure in the first three rows is the mean of two determinations. The gradients were 27-53%, and the density difference between adjacent fractions was 0.014 g/cc. Hence the values listed are only accurate within approximately ±0.007 g/cc.

<table>
<thead>
<tr>
<th>Buffer</th>
<th>Densities (g/cc) of Ribosomes and Subunits</th>
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<tbody>
<tr>
<td></td>
<td>74S</td>
</tr>
<tr>
<td>RSB</td>
<td>1.54</td>
</tr>
<tr>
<td>HMB</td>
<td>1.54</td>
</tr>
<tr>
<td>HMB + 50 mM EDTA</td>
<td></td>
</tr>
<tr>
<td>RSB (Huang and Baltimore, 1970)</td>
<td>1.54</td>
</tr>
</tbody>
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were VP0, VP1 and VP3 in an approximately equimolar ratio. The gel pattern of the particles was not consistent with contamination with soluble material. Treatment with ribonuclease, EDTA, LiCl and deoxycholate released the bulk of the labelled protein in a particle of about 6S.

(b) **Viral proteins attached to ribonucleoprotein complexes of 60-80S in infected cells**

The 70-80S regions of the sucrose gradients derived from infected cells, e.g. fractions 5-8 of Figure 11B, apparently contained labelled protein which could not be explained simply by a spreading of the adjacent peak of empty capsids. To determine the nature of the species present, appropriate fractions of these gradients were analysed by fixation with glutaraldehyde and centrifugation to equilibrium in caesium chloride density gradients. The densities of the ribosomes and subunits of uninfected cells as determined by Huang and Baltimore (1970) and in this work, are given in Table 2. However, some difficulties which were met with the fixation procedure are worthy of mention here as they reflect on results obtained in other laboratories. They are illustrated as follows.

In one experiment, $^{14}$C-labelled 74S ribosomes or 60S subunits of uninfected cells were added to portions of the $^3$H-labelled 60-80S region of infected cell gradients identical to that shown in Figure 11B, and the resulting mixtures fixed with glutaraldehyde. In this and subsequent experiments, the amount of ribosomal material fixed was calculated from the absorbance at 260 nm as described in...
Figure 20  Isopycnic centrifugation in CsCl after fixation with 8% glutaraldehyde of (A) 7 μg of uninfected 14C-labelled 74S ribosomes from the experiment of Figure 11A, 22 μg of the 74S 3H-labelled region from a gradient identical to that of Figure 11B and (B), 5 μg of uninfected 14C-labelled 60S subunits from the experiment of Figure 11A, 22 μg of the 60S 3H-labelled region from a gradient identical to that in Figure 11B.
Figure 21 A portion of the same material from the 74S region of a gradient of infected cell extract fixed in Figure 20A was co-electrophoresed with $^{35}$S-labelled naturally occurring empty capsids prepared as described in section 2.5(b) but labelled with $[^{35}$S]methionine.
section 2.4(a). Figures 20A and 20B show the density gradients after fixation of the 74S and 60S regions, respectively, using mixtures of $^{14}$C-labelled uninfected and $^{3}$H-labelled infected material. In both cases the majority of the $^{3}$H-labelled viral protein banded at a density of 1.47 - 1.48 g/cc, and there was no doubt that the material of this density contained VP0, VP1 and VP3. For example, a portion of the preparation from infected cells fixed in Figure 20A was co-electrophoresed with $^{35}$S-labelled empty capsids (Figure 21). The large proportion of $^{3}$H-radioactivity in VP0, VP1 and VP3 demonstrated the presence of these proteins in the 1.47 - 1.48 g/cc complex.

However, it was clear in Figure 20 that some of the added uninfected $^{14}$C-labelled ribosomes and subunits also banded at 1.47 - 1.48 g/cc, when in the absence of infected material a single peak at the density given in Table 2 was obtained (see later in Figure 23C). These observations suggested the possibility that the material at 1.47 - 1.48 g/cc contained a non-specific aggregate of ribosomal material and viral protein, representing an artifact caused by fixation. The viral protein was possibly free (density 1.30 g/cc) in the unfixed material, or complexed with ribosomal structures having a density other than 1.47 - 1.48 g/cc in the absence of aggregation. Before discussing the evidence that such structures did exist, some factors affecting the aggregating or "cross-fixing" of material will be described.
Figure 22  (A) 8 x 10^7 cells were labelled for 16 hr with 200 \( \mu c \) of \([^{3}H]\)uridine. A cytoplasmic extract was prepared, mixed with \(^{35}\)S-labelled empty capsids and centrifuged through a 15-30% sucrose gradient in RSB for 15 hr at 25,000 rpm in the Spinco SW 25.1 rotor. Portions of fraction 8 were fixed and analysed on CsCl gradients. (B) 33 \( \mu g \) of ribosomal material, 8% glutaraldehyde; (C) 8 \( \mu g \) of ribosomal material, 1.6% glutaraldehyde.
(i) **Cross-fixing with glutaraldehyde**

The following experiment demonstrated that naturally occurring empty capsids could also be fixed into material of density higher than that of protein. $8 \times 10^7$ cells were infected and treated as in Figure 11B, except that the cells were labelled with $[^{35}S]$methionine from 2.8 - 4.3 hours after infection. The sucrose gradient fractions constituting the peak of empty capsids at 65S were pooled, adjusted to 1 M NaCl, and centrifuged at 64,000 rpm for one hour in the Spinco SW 65 rotor. The pellet of empty capsids was resuspended in 10% CsCl, layered on a 15 - 40% CsCl gradient in RSB and centrifuged to equilibrium (Spinco SW 65 rotor, 59,000 rpm for 5 hours). The peak of radioactivity at the density of protein, 1.30 g/cc, was dialysed 24 hours against RSB. The preparation was mixed with a cytoplasmic extract of $8 \times 10^7$ uninfected cells labelled for 16 hours with 200 µc of $[^3H]$uridine, and the mixture sedimented through a 15 - 30% sucrose gradient in RSB (Figure 22A). The peaks of tritium coinciding with labelled ribosomal RNA were at 74S, 60S and 45S, i.e. fractions 7, 13 and 20 respectively. As expected, the $^{35}S$-labelled empty capsids sedimented between the 74S and 60S peaks. A portion of fraction 8 of the gradient, containing 33 µg of ribosome material, was fixed with 8% glutaraldehyde and analysed on a caesium chloride equilibrium gradient (Figure 22B). With complete resolution of the species originally added, peaks of tritium
Figure 23  Isopycnic centrifugation in CsCl after fixation with glutaraldehyde of $^{14}$C-labelled 60S ribosomal subunits and the $^{3}$H-labelled 60S region of infected cell extracts.

(A) 5 µg of $[^{14}C]$ material, 21 µg of $[^{3}H]$ material, 8% glutaraldehyde.
(B) 5 µg of $[^{14}C]$ material, 5 µg of $[^{3}H]$ material, 1.6% glutaraldehyde.
(C) 5 µg of $[^{14}C]$ material, 1.6% glutaraldehyde.
(D) 5 µg of $[^{3}H]$ material, 1.6% glutaraldehyde.
at 1.56 and 1.54 g/cc, and of $[^{35}S]$ at 1.30 g/cc would be expected. However, under these conditions the labelled protein was fixed into an aggregate of higher density (1.44 g/cc). Reducing the glutaraldehyde concentration to 1.6% and the amount of material fixed to 8 μg (Figure 22C) greatly decreased the amount of protein at higher densities, although there was still a proportion distributed at high densities throughout the gradient.

The effect of glutaraldehyde concentration and sample quantity on cross-fixing was further investigated using $^{14}$C-labelled 60S subunits from uninfected cells (fractions 12, 13 of Figure 11A) and the $^3$H-labelled 60S region of a gradient of infected cell extract (equivalent to fractions 11-13, Figure 11B). The samples fixed and centrifuged in caesium chloride gradients were (a) 5 μg of $[^{14}C]$ material, 21 μg of $[^{3}H]$ material and 8% glutaraldehyde, (b) 5 μg, 5 μg and 1.6% respectively, (c) 5 μg of $[^{14}C]$ subunits alone and 1.6% glutaraldehyde, (d) 5 μg of $[^{3}H]$ material alone and 1.6% glutaraldehyde (Figure 23).

Reducing the amount of viral protein material and the glutaraldehyde concentration increased the density of the major viral protein peak from 1.45 (Figure 23A) to 1.50 g/cc (Figure 23B), but this peak still contained $^{14}$C-labelled subunits which by themselves banded at the expected density of 1.56 g/cc (Figure 23C). Even at the lowest concentration of viral protein fixed (Figure 23D) there was still a peak at 1.50 g/cc. The proportions of $^3$H-radioactivity recovered in the 1.45/1.50 peaks of
Figures 23A, 23B, 23D were closely similar (69%, 81% and 80%, respectively). A comparison of Figures 23B and 23D led to the conclusion that the peak at 1.50 g/cc contained at least some cross-fixed ribosomal material, but that the amount of viral protein cross-fixed was not increased by the presence of excess ribosomes. However, it was not possible to say to what extent the cross-fixation was a result of the addition of glutaraldehyde, or whether there were components of the $^3$H- and $^{14}$C-labelled samples that interacted before fixation.

The lesson learned from these results was that identification of species by density in complex mixtures was not valid unless the effects of cross-fixation were controlled. One practical conclusion was that there was only one satisfactory way of demonstrating, by these techniques, the existence in infected cell extracts of ribosomal complexes carrying VP0, VP1 and VP3. This was to fix, and analyse on caesium chloride gradients, sucrose gradient fractions that contained material greater than 60S in sedimentation coefficient, and in which the possibility of empty capsids being fixed into material of density higher than protein could be excluded. For this purpose the following experiments were done.

(ii) Evidence for ribosomal complexes containing viral protein

Infected cells were labelled with a mixture of $[^3]$H-leucine and $[^3]$H-lysine for a shorter time (3 - 3.7 hours after infection) to reduce the proportion of empty
Figure 24 A cytoplasmic extract of $4 \times 10^7$ infected cells treated as in Figure 11B, but labelled from 3.0 to 3.7 hr after infection with 200 $\mu$C of a 2:1 mixture of [$^3$H]leucine and [$^3$H]lysine, was layered on a 15-30% sucrose gradient in RSB and centrifuged 16.5 hr at 22,000 rpm in the Spinco SW 25.1 rotor.
Figure 25  Isopycnic centrifugation in CsCl after fixation with 1.6% glutaraldehyde of (A) 15 μg of fractions 10-12 of Figure 24; (B) 15 μg of fractions 10-12 of Figure 24 and a 35S-labelled mixture of virus and empty capsids; (C) the same 35S-labelled mixture used in (B).
Figure 26  Co-electrophoresis of $^{35}$S-labelled empty capsids (prepared as in section 2.5(b) using $[^{35}\text{S}]$methionine) and a portion of the same 60-70S material used for Figures 25A and 25B prepared for electrophoresis by precipitation with TCA [section 2.6(a)].
capsids in the cytoplasmic extract, and analysed on a 15 - 30% sucrose gradient as in Figure 11. The result is shown in Figure 24. A mixture was made of virus prepared as in section 2.5(a) but labelled with \[^{35}\text{S}]\text{methionine}, and \[^{35}\text{S}]\text{methionine}-labelled empty capsids prepared as described in the first part of the immediately preceding section. Virus was included because the fractions obtained from the bottom of sucrose gradients sometimes contained virus particles (Figures 11B and 12), and it was possible that these virions contributed to the cross-fixing.

Fractions 10, 11, 12 of the sucrose gradient shown in Figure 24 (60-70S) were pooled, and 15 µg of ribosomal material fixed with 1.6% glutaraldehyde in the absence and presence of a sample of the \[^{35}\text{S}]\text{-labelled mixture, then centrifuged in a caesium chloride gradient (Figure 25). The addition of the \[^{35}\text{S}]\text{-labelled mixture did not alter the fixation pattern. In Figure 25A, 44\% of the }^{3}\text{H}-\text{radioactivity was recovered between fractions 3 and 9. The value for the identical region in Figure 25B was 43\%, and it contained no peak of }^{35}\text{S-label. Thus there was no cross-fixation of empty capsids or virions in this experiment. The composition of the }^{35}\text{S}-\text{labelled mixture resolved in a 22-38\% CsCl gradient is shown in Figure 25C.}

A portion of the \(^{3}\text{H}-\text{labelled material illustrated in Figures 25A and 25B was co-electrophoresed with }^{35}\text{S}-\text{labelled empty capsids (Figure 26); the distribution of the peak heights in empty capsids with methionine label differs from that with RPH label (Figure 9A), VP3 being
Figure 27  Isopycnic centrifugation in CsCl after fixation with glutaraldehyde of the EDTA-treated material of 60-80S from infected cell extracts. (A) 15 µg of another portion of the same material used in Figures 25A and 25B, 1.6% glutaraldehyde. (B) 22 µg of another portion of the same material used in Figure 20A, 8% glutaraldehyde.
predominant. The relative heights of the VP0, VP1 and VP3 peaks in Figure 26 for the \[^{3}H\]lysine/\[^{3}H\]leucine label were similar to those in Figure 9A for \[^{3}H\]RPH. As 71% of the recovered \(^3\)H-radioactivity migrated between fractions 35 and 56 in Figure 26, it was apparent that the polypeptides of this part of the gel were present in the complexes of 1.50 - 1.54 g/cc shown in Figures 25A and 25B.

To ascertain the nature of the ribosomal complexes carrying viral polypeptides, an identical portion of the material used for Figure 25A was treated with 20 mM EDTA before fixation and density gradient centrifugation (Figure 27A). The proportion of label at density 1.50 - 1.54 g/cc decreased from 44% to approximately 21%, most apparently being converted to protein (1.30 g/cc) but some appeared at a density of 1.40 g/cc, the density of viral ribonucleoprotein, vRNP (Baltimore and Huang, 1970; Huang and Baltimore, 1970). A portion of the tritiated sample from the 74S region of the infected cell extract used for Figure 20A was also treated with EDTA at 50 mM, a concentration which is known to destroy viral polysomes completely (Miller, 1972). A peak at the density of vRNP (1.39 g/cc) with a shoulder at the density of the small ribosomal subunit (in EDTA, 1.43 g/cc - Table 2) was obtained (Figure 27B), although the bulk of the label banded at the density of protein (1.31 g/cc).

These experiments suggested the existence of a ribonucleoprotein complex containing vRNP and the viral proteins VP0, VP1 and VP3, together with either a small
ribosomal subunit or at least one complete ribosome. Huang and Baltimore (1970) proposed that a vRNP-small subunit complex had a density of 1.44 g/cc and a vRNP-ribosome complex a density of 1.47 g/cc. The 1.47 complex obtained in the work described in this thesis contained some cross-fixed material, a finding which perhaps casts some doubt on the validity of the conclusion of Huang and Baltimore (1970) that the 1.44 and 1.47 complexes were polyribosome precursors in vivo. On the other hand, it is not certain that all the 1.47 complex found in section 3.3(b)(i) was the result of the fixation procedure. It is most likely that the 1.50 - 1.54 g/cc and 60-70S components of Figures 25A and 25B consisted of vRNP complexed with one, or at the most two, ribosomes. The possibility that these components contained a complex of vRNP (density 1.40 g/cc) and only a small ribosomal subunit (density 1.48 g/cc) is unlikely, as such a complex is not expected to have a density higher than either of its constituents. Some of the material of density 1.54 g/cc may have represented free 74S ribosomes that had VP0, VP1 and VP3 attached to the small subunit.

In summary, this section demonstrated that, when cross-fixation artifacts were avoided, viral protein could be shown to be attached to particles of 60-70S with a density of 1.50-1.54 g/cc. These structures were labile to EDTA, with most viral protein being converted to free protein (1.30 g/cc) but some remaining at a density of 1.40 g/cc. These particles were likely to comprise strands of vRNP
Figure 28  The procedure was similar to that in Figure 11C. 4 x 10^7 cells were infected with Mahoney virus, incubated 3 hr, and then guanidine hydrochloride added to a final concentration of 2 mM. Ten min later, [35S] methionine was added to a final concentration of 10 μc/ml. The cells were incubated for a further 2 hr. The cytoplasmic extract was centrifuged through a 15-30% sucrose gradient in RSB for 14.5 hr at 21,000 rpm in the Spinco SW 27 rotor.
Figure 29  

$^3$H-labelled Mahoney virions prepared as in section 2.5(a) were co-electrophoresed with a portion of fractions 9-11 of Figure 28 prepared for electrophoresis by precipitation with TCA [section 2.6(a)].
complexed to a small number of ribosomes, i.e. incipient polyribosomes. The attached viral protein was again VP0, VP1 and VP3 in approximately equimolar ratios.

(c) The effect of guanidine on ribonucleoprotein complexes carrying viral protein

Because guanidine inhibits the growth of poliovirus (Appleyard, 1967; Lwoff, 1965) and the locus for guanidine resistance of poliovirus maps in the structural protein region (Cooper, Wentworth and McCahon, 1970), it was of interest to examine the effect of guanidine on the viral protein-ribosomal complexes found in infected cells and described in the previous section. The cytoplasm of cells labelled with $[^3H]$RPH from 3 to 5 hours after infection in the presence of guanidine typically gave a sucrose gradient pattern (Figure 11C) different from that of cytoplasm labelled without guanidine (Figure 11B). One of three repeats of the experiment of Figure 11C is shown in Figure 28. The only difference was the use of $[^3S]$ methionine as label. The result was always the same. There was no peak of acid-insoluble radioactivity at 45S, nor an obvious shoulder at 70-80S. There was a substantial peak at 60-65S as shown by Jacobson and Baltimore (1968b), although a value of 74S for this peak was indicated from their gradients which spanned a wider range of S values. This peak at 60-65S corresponded in sedimentation constant and polypeptide composition to empty capsids, consisting exclusively of VP0, VP1 and VP3 (Figure 29).
Figure 30  (A) Isopycnic centrifugation after fixation with 8% glutaraldehyde of 11 μg of fractions 5 and 6 of Figure 28. (B) Co-electrophoresis of a portion of fractions 5 and 6 (TCA precipitated) of Figure 28 with Mahoney ³H-labelled virions.
Fractions 5 and 6 from the 70-80S region of the gradient shown in Figure 28 were pooled and 11 μg of ribosomal material fixed with glutaraldehyde. Subsequent centrifugation on a caesium chloride gradient (Figure 30A) showed that the major component present had a density of 1.40 g/cc, the density of vRNP, and contained 47% of the protein label in the gradient. The gel pattern given in Figure 30B of another portion of the material used for the fixation gradient (Figure 30A) showed that 64% of the radioactivity was recovered in the polypeptides VP0, VP1 and VP3 (fractions 26-44). This suggested that the vRNP in the sample analysed contained at least some of these three polypeptides. Density gradient analysis after fixation with glutaraldehyde of the 60-65S material showed a single peak at 1.47 g/cc, but the opportunity was not available in this instance to examine the effects of cross-fixation.

Thus the main finding of this section was that guanidine appeared to inhibit the formation of the two species of ribosome-viral protein complexes described in sections 3.3(a) and 3.3(b) of this chapter. The native 45S subunits no longer carried VP0, VP1 and VP3, and in place of an obvious shoulder of ribosome-vRNP complex at 70-80S, there was only a small amount of material, most of which had a density of 1.40 g/cc corresponding to vRNP. There was sufficient VP0, VP1 and VP3 in this preparation, however, to make it fairly certain that the vRNP did contain these proteins.
Figure 31 5 x 10^7 cells were infected with Mahoney virus, incubated 3 hr in Auto-Pow medium, resuspended in Auto-Pow with 100 μc of [³H]uridine at a concentration of 4 x 10^6 cells/ml, and incubated 2 hr. The cytoplasmic extract was centrifuged through a 15-30% sucrose gradient in RSB for 13.5 hr at 24,000 rpm in the Spinco SW 25.1 rotor. 0.1 ml of each fraction was tested for resistance to ribonuclease as described in the text.
Figure 32  Isopycnic centrifugation in CsCl after fixation with 8% glutaraldehyde of 28 µg of a mixture of $^{14}$C-labelled 45S ribosomal subunits from Figure 11A and fraction 17 of Figure 31. The refractive indices of fractions were determined after the fractions had partly evaporated. These measurements confirmed the linearity of the gradient but prevented accurate estimations of densities.
(d) Ribonucleoprotein complexes in infected cells labelled with $[^3H]$uridine

It was desirable to confirm that labelled viral RNA could be found in 60-80S material of density 1.50-1.54 g/cc comparable to the protein labelled complexes found in infected cells [section 3.3(b)(ii)], and that such material did have the properties of vRNP-ribosome complexes. This was done by using a uridine label in experiments similar to those already described. The procedure was as follows.

Cells were labelled with $[^3H]$uridine at 8 $\mu$C/ml from 3 to 5 hours after infection, a period when virtually all RNA synthesis was viral. Actinomycin D was not used to completely inhibit host RNA synthesis in these experiments. The cytoplasmic extract of one experiment analysed on a sucrose gradient as in Figure 11B is shown in Figure 31. In addition, 0.1 ml of each gradient fraction was treated with 1 $\mu$g of ribonuclease for 10 minutes at 37°C before placing on the Whatman discs for acid washing. The large peak of acid-insoluble and ribonuclease-resistant material at approximately 20S (fractions 23-25, Figure 31) corresponded to viral double-stranded RNA. The slight peak at 45S (fraction 17) resulted from some residual labelling of ribosomal RNA, shown by mixing fraction 17 with 45S ribosomal subunits of uninfected cells labelled with $^{14}$C amino acids, fixing and centrifuging to equilibrium in a caesium chloride gradient (Figure 32). Both preparations of subunits had the same density.
Figure 33 Isopycnic centrifugation in CsCl after fixation with 8% glutaraldehyde of (A) 42 μg of a mixture of \(^{14}C\)-labelled 74S ribosomes and the \(^3H\)uridine-labelled fractions 7,8 of Figure 31; (B) 25 μg of a similar mixture, but the infected material from a replicate experiment; (C) 5 μg of the 70-80S material from infected cells treated with EDTA.
As expected, problems of cross-fixing were encountered when a total of 42 µg (ribosomal material) of a mixture of the $[^3H]$uridine-labelled 70-80S peak from infected cells (fractions 7,8 Figure 31) and uninfected $^{14}\text{C}$-labelled 74S ribosomes (from the experiment of Figure 11A) were fixed and analysed on a caesium chloride gradient (Figure 33A). The $[^3H]$uridine-labelled material banded at densities of 1.53 g/cc and 1.47 g/cc. The $^{14}\text{C}$-labelled ribosomes not only gave a peak at 1.55 g/cc, i.e. close to their correct density (Table 2), but also a smaller peak at 1.47 g/cc, the density characteristic of cross-fixed material (Figure 20). Subsequently, fixation of a total of 25 µg of a similar mixture using infected material from an identical replicate experiment gave only one $[^3H]$uridine-labelled peak at 1.53 g/cc (Figure 33B). The marker $^{14}\text{C}$-labelled ribosomes remained at a density of 1.54 g/cc, indicating a lack of cross-fixation.

The $[^3H]$uridine-labelled material banding at 1.53 g/cc corresponded in density to viral monoribosomes or polyribosomes (Huang and Baltimore, 1970; Miller, 1972). Treatment of the material with 50 mM EDTA before fixation dislodged the ribosomes and converted all the $[^3H]$uridine-labelled material to the density of vRNP, 1.41 g/cc (Figure 33C).

Thus the experiments described in this section demonstrated the existence of viral RNA-containing structures sedimenting at 70-80S, which had a density of
Figure 34  (A) A portion of the 70-80S region from a gradient similar to that in Figure 11B was treated with ribonuclease, LiCl as described in the text and centrifuged through a 15-30% sucrose gradient in RSB for 5.5 hr at 24,500 rpm in the Spinco SW 25.1 rotor at 5°C.  (B) A portion of the 60-70S region from a gradient similar to that in Figure 28 was treated with ribonuclease, NaCl as described in the text and centrifuged through a 25-40% sucrose gradient for 3 hr at 20°C at 64,000 rpm in the Spinco SW 65 rotor.  Buffer for the gradient was 10 mM Tris HCl pH 7.3, 0.2 M NaCl and 0.1% sodium deoxycholate.  Fraction 1 was a pad of 60% sucrose dissolved in this buffer.
1.53 g/cc, and which were disrupted by EDTA to yield only one [³H]uridine-labelled component of the density of vRNP. These structures therefore had the properties of ribosome-vRNP complexes labelled only in the viral RNA.

(e) Attempts to dislodge VP0, VP1 and VP3 from the 60-80S ribonucleoprotein complexes

A discrete particle was obtained from disrupted 45S subunits (Figure 17), with a sedimentation constant of 6S that was consistent with a polypeptide composition of one molecule each of VP0, VP1 and VP3. In view of this success, attempts were made to dislodge similar particles from protein-labelled 60-80S material. The 60-80S region of the gradients of infected-cell extracts was potentially a rich source of such particles because it contained more radioactivity than the 45S region, and approximately 50% of this label was in ribonucleoprotein complexes carrying VP0, VP1 and VP3.

The attempts were, on the whole, unsuccessful. Figure 34A shows the result of treating protein-labelled material of 70-80S from a gradient similar to that of Figure 11B with 10 µg/ml of ribonuclease for 10 minutes at 37°C, and then adjusting it to a concentration of 2 M LiCl. The sample was diluted with an equal volume of RSB and centrifuged through a 15-30% sucrose gradient in RSB. The majority of the labelled protein sedimented in a broad band between approximately 80S and 160S. The sedimentation
Figure 35 Co-electrophoresis with $^{3}$H-labelled Mahoney empty capsids of (A) the 85S peak of Figure 34B and of (B) the 65S peak of Figure 34B. The $^{35}$S-labelled material was prepared for electrophoresis by precipitation with TCA and resuspension in SUMP [section 2.6(a)].
constants were calculated as described by McEwen (1967). Apparently the disrupted ribosome-ribonucleoprotein complexes had reaggregated into structures with a range of sedimentation constants.

A second experiment was done using the 60-70S region of a gradient identical to that shown in Figure 28. This time the sample was treated with 20 μg/ml of ribonuclease and adjusted to 0.2 M NaCl. The treated preparation was centrifuged through a 25-40% sucrose gradient in 10 mM Tris HCl pH 7.3, 0.2 M NaCl and 0.1% sodium deoxycholate (Figure 34B). Approximate sedimentation constants of the peaks obtained were calculated as above. The gel patterns of the two main peaks are shown in Figure 35. Both consisted almost entirely of VP0, VP1 and VP3. The most plausible explanation of these results was that the peak at 65S comprised the empty capsids in the sample, which were resistant to the treatments, and that the other peak was composed of aggregated [VP0, VP1, VP3] monomers, possibly in a complex of 6S. The reason for the lack of aggregation after the disruption of 45S subunits (Figure 17) was probably the much lower concentration of material in that preparation. The self-assembly to empty capsids of 14S structural precursors consisting of VP0, VP1 and VP3 is known to be concentration dependent (Phillips, 1969, 1971). Thus disruption of 60-80S material from infected cells with ribonuclease and high salt concentrations
Figure 36  3.2 x 10^8 cells were infected with Mahoney virus and incubated for 2.75 hr in Auto-Pow medium, then for 0.5 hr in Eagle's medium lacking amino acids. Both media contained 0.2 μg/ml actinomycin D. The culture was split in two, and guanidine hydrochloride added to one portion to a final concentration of 2 mM. Five min later, 200 μc of [3H]uridine was added to both cultures. After a 15 min incubation, puromycin was added to a final concentration of 100 μg/ml. Five min later, the cells were chilled and cytoplasmic extracts prepared. P20 pellets were resuspended in RSB, deoxycholate/Brij added to 1% each, and then centrifuged at 20,000 rpm for 1.5 hr through separate 15-30% sucrose gradients in RSB in the Spinco SW 25.3 rotor.
failed to dislodge a particle with the sedimentation constant expected of an aggregate of one molecule each of VP0, VP1 and VP3.

3.4 Viral Proteins in the Guanidon and the Replication Complex

The replication complex in the cytoplasm of infected cells contains replicase(s) and replicative intermediate, and sediments heterogeneously at approximately 250S (Girard, Baltimore and Darnell, 1967; Caliguiri and Mosser, 1971). In the presence of guanidine, newly synthesized RNA remains in a larger structure named the guanidon, which has a sedimentation constant much greater than normal replication complex (Baltimore, 1968). Since it is proposed that a regulator particle has an affinity for the 5' end of vRNP, and it is an increase in this affinity in the presence of guanidine that leads to the formation of the guanidine by preventing the release of RNA molecules (Cooper et al., 1973; see Chapter Six), the polypeptide composition of the guanidon and replication complex fractions of infected cells was analysed.

(a) Labelling with $[^3H]$uridine: confirmation of the guanidon

The general procedure used in several experiments is described in the legend of Figure 36. Briefly, cells were labelled with $[^3H]$uridine for 15 minutes at 3.3 hours after infection in the presence and absence of guanidine, and then treated for 5 minutes with
Figure 37 Isopycnic centrifugation in CsCl after fixation with 8% glutaraldehyde of a portion of fractions 5-12 of the gradient in Figure 36 derived from guanidine-treated cells. (A) Fixation in RSB. (B) Fixation after treatment with 20 mM EDTA.
puromycin to destroy the polysomes. P20 pellets (Girard et al., 1967) were prepared by centrifuging the cytoplasm for 30 minutes at 15,000 rpm in the Spinco SW 50.1 rotor. The pellets were resuspended in RSB, treated with detergent, and analysed on separate gradients (Figure 36). The main peak in the extract labelled without guanidine was at approximately 70-150S (fractions 23-27), together with another broad peak around fractions 14 and 15. In the extract of cells treated with guanidine, there was a broad peak at approximately 1000S (fraction 9), confirming the report of Huang and Baltimore (1970) regarding the sedimentation characteristics of the guanidon.

Fractions 5 to 12 of each gradient of Figure 36 were pooled, i.e. the guanidon region from infected cells treated with guanidine, and that part of the replication complex region (from untreated cells) which sedimented at higher than average S values. A portion of the pooled fractions was fixed with glutaraldehyde and centrifuged to equilibrium in caesium chloride gradients. In each case a single peak at a density of 1.54 g/cc was obtained, although the guanidine-treated sample only is shown in Figure 37A. In addition, the guanidine-treated preparation was adjusted to 20 mM EDTA before fixation (Figure 37B). Most of the radioactivity was recovered at a density of 1.44 g/cc with a much smaller amount at 1.40 g/cc.

The previously reported density of the guanidon fixed in RSB was 1.44 g/cc (Huang and Baltimore, 1970), and for the replication complex fixed in either RSB or in the
Figure 38  3.2 x 10^8 cells were infected with Mahoney virus and incubated for 2.75 hr in Auto-Pow medium containing 0.2 μg/ml actinomycin D. The cells were resuspended in Eagle's medium lacking amino acids but containing [³⁵S]methionine at 12 μc/ml and actinomycin D at 0.2 μg/ml. Thirty min later, the culture was split in two and guanidine hydrochloride added to one portion to a final concentration of 2 mM. After 20 min, puromycin was added to 100 μg/ml to both cultures, which were then incubated a further 5 min. P20 pellets were prepared and analysed on separate sucrose gradients as in Figure 36.
presence of EDTA was also 1.44 g/cc (Baltimore and Huang, 1968). The higher density of 1.54 g/cc found in the present work may result from an association of the replication complex with ribosomes, or because the structure contains double-stranded regions and correspondingly less attached protein. An alternative explanation for the high density, that the material fixed comprised entirely polyribosomes, could be excluded for two reasons. Firstly, the cells were treated with puromycin at a concentration (100 µg/ml) known to break down polysomes (Latham and Darnell, 1965). Secondly, the cells were incubated in 0.2 µg/ml actinomycin D for 3.5 hours before labelling and hence the only label in polysomes could have been in viral RNA. If the puromycin treatment had failed for some reason, the addition of EDTA would have given a band at density 1.40 g/cc.

(b) Labelling with \([^{35}\text{S}]\)methionine and the probable presence of VP0, VP1 and VP3 in the replication complex and guanidinon

Figure 38 shows sucrose gradients of P20 pellets prepared from cells labelled with \([^{35}\text{S}]\)methionine in the presence and absence of guanidine. The experimental design was similar to that used for Figure 36, except that the \([^{35}\text{S}]\)methionine was added 2.75 hours after infection, 30 minutes before the addition of guanidine. Less label was incorporated into the cells treated with guanidine, and in contrast to the results with uridine label, there was no increase in radioactivity in the guanidinon region of the
Figure 39  Isopycnic centrifugation after fixation with 8% glutaraldehyde of a portion of fractions 4-14, Figure 38 from (A) cells treated with guanidine, (B) cells without guanidine.
Figure 40  Co-electrophoresis of $^3$H-labelled empty capsids with fractions 4-14, Figure 38 from (A) cells treated with guanidine, (B) untreated cells. The $^{35}$S-labelled material was prepared for electrophoresis by precipitation from the sucrose gradient fractions with TCA [section 2.6(a)].
Figure 41  Gel electropherograms of P20 pellets and supernatants of cytoplasmic extracts prepared from cells treated as in Figure 38. Material was prepared for electrophoresis by TCA precipitation as in section 2.6(a). (A) Pellet, cells treated with guanidine. (B) Supernatant, cells treated with guanidine. (C) Pellet, no guanidine. (D) Supernatant, no guanidine. ^3^H-labelled empty capsids were added to the sample electrophoresed in (C).
gradient. Nevertheless, fractions 4-14 of each gradient were pooled, a portion of each fixed and centrifuged in caesium chloride (Figure 39) and the remainder co-electrophoresed with $^3$H-labelled empty capsids (Figure 40). In Figure 40B, 63% of the recovered radioactivity was in the VPO, VP1 and VP3 region while in Figure 39B, 63% of the radioactivity was in the component of density 1.54 g/cc, the density of the replication complex and guanidon in these experiments. This suggested that the replication complex contained some VPO, VP1 and VP3. Since the identical comparison for Figures 40A and 39A gave values of 45% and 56%, a similar conclusion for the guanidon was likely but not certain.

In order to see what polypeptides were enriched in the sucrose gradient fractions pooled, portions of the P20 pellets and supernatants from guanidine-treated and untreated infected cells were electrophoresed (Figure 41). Compared with the P20 supernatant material (Figures 41B and 41D), the guanidon (Figure 40A) and replication complex (Figure 40B) contained relatively more NCVP4, but less NCVP1 and NCVP2. The most obvious difference between the P20 pellets (Figures 41A and 41C), and the guanidon and replication complex (Figures 40A and 40B), was the presence in the former of a third polypeptide, possibly NCVPX (Jacobson and Baltimore, 1968a; Jacobson et al., 1970) in the VPO, VP1 region of the gel. This area was satisfactorily resolved for the added $^3$H-labelled empty capsids (Figure 41C).
Figure 42  10-30% sucrose gradients centrifuged in parallel for 4.7 hr at 26,000 rpm in the Spinco SW 27 rotor. The buffer in (A) and (B) was 10 mM Tris HCl pH 7.4, 100 mM NaCl and 0.5% Brij. In (C) it was RSB. (A) A portion of fractions 4-14 of a gradient identical to that in Figure 38 (no guanidene) treated with ribonuclease, CsCl, as described in the text. The S value of the main peak was calculated by the method of McEwen (1967). (B) A similar preparation to that in (A), derived from cells treated with guanidene. (C) Empty capsids, prepared from a CsCl gradient as in section 2.5(b).
but not for the $^{35}$S-labelled preparation. It was probable that at least some of the proteins present in the gels of Figure 40 represented replicase proteins, and NCVP2 (fraction 15 in 40A, 16 in 40B) has been previously implicated as a replicase protein [discussed in section 1.1(b)]. Another possible candidate was NCVP4 with an approximate molecular weight of 45,000 daltons (fraction 27 in 40A, 26 in 40B).

(c) Release of VP0, VP1 and VP3 from the replication complex

In an attempt to determine the nature of the large (200-1000S) structure containing VP0, VP1 and VP3, fractions 4-14 of gradients identical to those shown in Figure 38 were pooled, treated at room temperature for one hour with 5 µg/ml of ribonuclease, and made 0.5% in Brij 58 in a volume of 5 ml. 2.5g of caesium chloride was dissolved in each preparation, and the solutions centrifuged at 48,000 rpm for 20 hours in the Spinco SW 65 rotor. All the radioactivity recovered in each gradient was in the top two fractions. These were pooled, dialysed for one hour against 10 mM Tris HCl pH 7.4, 100 mM NaCl, 0.5% Brij 58 and then centrifuged through 10-30% sucrose gradients in the same buffer (Figure 42), in comparison with a preparation of empty capsids.

It is clear from Figure 42 that empty capsids were not the major proteinaceous structure in replication complex from cells either treated with guanidine or untreated. The gel pattern of the 100-120S peak, fractions
Figure 43  Co-electrophoresis of the 100-120S peak in Figure 42A with $^3$H-labelled Mahoney empty capsids. Fractions 2-6 of Figure 42A were prepared for electrophoresis by precipitation with TCA as described in section 2.6(a).
2-6 of Figure 42A, is shown in Figure 43. Once again, VP0, VP1 and VP3 were the major polypeptides present, although the existence of VP2 suggests that some VP0 was already cleaved. Because of insufficient radioactivity, the 100-120S region of Figure 42B was not electrophoresed, but in view of the overall similarity of the gel patterns in Figures 40A and 40B, it almost certainly contained some of the same polypeptides as the 100-120S structure in Figure 42A. The gel pattern of the material at the top of the gradient in Figure 42B (fractions 25-30) is not shown, but was found to contain mostly polypeptides of molecular weight greater than VP0.

Thus the experiments described in this section confirmed the existence of the guanidon at large S values, but demonstrated a different density (1.54 g/cc) for the guanidon and replication complex from that previously reported (1.44 g/cc). The replication complex and very probably the guanidon contained VP0, VP1 and VP3. In this case, as for the 60-80S ribosomal complexes [section 3.3 (e)], attempts to recover a particle of 5-7S failed despite the presence of VP0, VP1, and VP3. Instead a presumed aggregate was obtained, containing these three polypeptides, but not having the S value of naturally occurring empty capsids.
Figure 44  Total protein synthesis in infected and uninfected cells, treated with guanidine carbonate and actinomycin D as described in the text. The incorporation of \( [{}^3\text{H}] \)leucine into acid-insoluble material was determined as described in section 2.7.
3.5 The Inhibition of Ribosomal Protein Synthesis by Infection with Poliovirus

The labelling periods of the experiments described so far in this chapter were in the last half of the growth cycle, usually from three hours after infection. The purpose of leaving the addition of radioactive amino acids to this time was to reduce the labelling of host-coded proteins as much as possible, thus enabling viral polypeptides to be detected more readily. Since ribosomal fractions were specifically examined for viral proteins, it was desirable to be sure that the labelling of host ribosomal proteins was adequately suppressed. This section therefore describes the relationship between the inhibitions of total and ribosomal protein synthesis that occur during the first two hours after infection, prior to the start of the main period of viral RNA and protein synthesis.

(a) Inhibition of total protein synthesis

The data presented in Figure 44 are similar to those of Penman and Summers (1965) for the decline of host protein synthesis in poliovirus-infected cells. $9 \times 10^7$ cells were infected with $ts^+$ virus at an input multiplicity of 50 pfu/cell, and then incubated for two hours in medium lacking leucine but containing actinomycin D (0.5 µg/ml) and guanidine carbonate (200 µg/ml). The cells were washed twice with PBS (reversal), and resuspended in medium containing 0.5 µc/ml of $[^3H]$leucine as the only source of this amino acid.
Figure 45 4 x 10^7 cells were labelled as described in the text for 2 hr after reversal of guanidine inhibition and the cytoplasmic extracts treated by sedimentation through a sucrose cushion as described in section 2.4(a). The pellet was resuspended and centrifuged for 15 hr at 24,000 rpm through a 15-30% sucrose gradient in NEBl contained in the Spinco SW 25.1 rotor. (A) Uninfected cells. (B) Cells infected with ts+.
After two hours the infected cells had incorporated only 25% of the amount of \( {^3H}\)leucine incorporated by uninfected cells. Considering only the label incorporated by infected cells during the first two hours, 71% was incorporated during the first hour and 29% in the second. It was this decline in host protein synthesis that enabled viral protein synthesis to be detected (Summers et al., 1965).

(b) Inhibition of ribosomal protein synthesis

4 x \( 10^7 \) cells were infected and labelled with \( {^3H}\)leucine using the same procedure as described for Figure 44 except that the incubation was in medium containing 6 \( \mu \)c/ml for only the first two hours after reversal. The cells were then chased for 10 minutes with a 1,000 fold excess of leucine to remove nascent protein from the ribosomes before preparation of a cytoplasmic extract, which was given the preliminary centrifugation through the sucrose cushion as described in section 2.4(a). The pellet was resuspended in NEB 1 to convert all ribosomal material (polyribosomes, ribosomes and native subunits) to particles sedimenting at 50S and 30S, and centrifuged through a 15-30% sucrose gradient in NEB 1. Figures 45A and 45B show the gradients obtained from uninfected and infected cells respectively.

The peak fractions were pooled, the ribosomal material centrifuged to a pellet, heated in SUMP buffer and co-electrophoresed with \( ^{14}C\)-labelled ts\(^+\) virion proteins.
Figure 46  Polypeptides of the 30S subunits co-electrophoresed with the proteins of $ts^+$ virions prepared as in section 2.5(a). The 30S subunits for electrophoresis were obtained by centrifugation [section 2.6(a)] from the gradients in Figure 45. (A) Uninfected cells. (B) Infected cells.
Figure 47  Polypeptides of the 50S subunits were co-electrophoresed with $^{14}$C-labelled ts$^+$ virion proteins. The positions of VP2 and VP3 are indicated. The 50S subunits were obtained by centrifugation [section 2.6(a)] from the gradients in Figure 45. (A) Uninfected cells. (B) Infected cells.
Table 3A. The inhibition of ribosomal protein synthesis by infection.

The method used to obtain the above data from Figure 45 is described in the text.
Figures 46A and 46B are the gels obtained from the 30S subunits; Figures 47A and 47B are the gels for the 50S subunits. The gel patterns from infected cells compared with those from uninfected cells certainly showed that ribosomal protein synthesis was reduced as a result of infection. No significant new peak was detected with the time of labelling used in the infected cells, but there were minor differences in the relative heights of peaks also present in uninfected cells. In particular, the peak at fractions 12, 13 in Figure 46B probably corresponded to that at fraction 10 in Figure 19, although the experiments described in sections 3.3(a) and 3.3(b) used a later time of labelling and therefore labelling of host protein was even more suppressed.

Using the data in Figures 44 and 45 it was possible to show that the synthesis of ribosomal proteins, as a subclass of all the proteins being made by the cells, was inhibited at the same rate as the total protein synthesis of the cells (Table 3). It was also shown that there was no preferential inhibition of synthesis of the proteins found on the small or large ribosomal subunit.

Table 3A was obtained by adding the absorbance (A) at 260 nm and the acid-insoluble radioactivity in Figures 45A and 45B for the six fractions corresponding to the 50S peak, and the four fractions corresponding to the 30S peak. The recoveries of $A_{260}$ were similar for both gradients. The inhibitions by infection of 50S ribosomal
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Table 3B. A comparison of the inhibition of ribosomal protein synthesis and total protein synthesis in cells infected by poliovirus. To obtain the ribosomal protein data, 8 x 10^7 cells were infected, then divided into two cultures and treated as in Figure 45 except that the labelling period (4 μc/ml of [3H]leucine) was from 0 to 1 hrs after reversal of guanidine inhibition for one culture, and from 1 to 2 hrs after reversal for the other.
protein synthesis (70%), of 30S ribosomal protein synthesis (73%), and of total protein synthesis (75% from Figure 44) during the first two hours after guanidine reversal were also similar.

Table 3B was derived from gradients similar to those of Figure 45 in which cytoplasmic extracts were made from infected cells labelled 0 to 1 and 1 to 2 hours after reversal of guanidine inhibition. The amounts of label incorporated into 50S and 30S protein over each time period were calculated as for Table 3A, and compared with the total protein synthesis measured in the earlier experiment shown in Figure 44. The figures in the last row of Table 3B are the sums of the first two rows, and were taken as 100% for calculation of the percentages in the last three columns. Thus the decline in both 50S and 30S ribosomal protein synthesis paralleled the decline in total protein synthesis of infected cells.

By assuming that all ribosomal material in a cell homogenate was recovered in the two gradient peaks, it was calculated that the synthesis of proteins associated with the EDTA-treated ribosomes constituted only 12% of the total protein synthesis in both infected and uninfected cells over the 2 hour period following reversal. This meant that the parallel decline in total protein synthesis and the synthesis of proteins found in EDTA-treated ribosomes was not simply due to the latter constituting the majority of the total cell protein synthesis.
Figure 48  A diagrammatic interpretation of the results presented in section 3.3. Because almost all CsCl gradients were 53-27% w/w, the densities are only accurate to approximately ±0.007 g/cc
3.6 Discussion

Figure 48 summarizes some characteristics of the ribosomal complexes that have been deduced to occur in these experiments and that will be referred to in the following discussion.

(a) The small ribosomal subunit

The presence of a 6S particle on the small ribosomal subunits obtained from poliovirus-infected cells and carrying the viral proteins VP0, VP1 and VP3 (A of Figure 48) is consistent with the concept that this particle is responsible for the switch in protein synthesis from host to virus-coded. This finding is one of several lines of experimental support for the equestron hypothesis, which is discussed in section 1.1(e) and in Chapter Six of this thesis.

It has been pointed out in section 1.2 that initiation of translation seems to be the point at which the inhibition of host protein synthesis is effected in poliovirus-infected cells. Less indirect evidence that a particle comprising VP0, VP1 and VP3 actually has the activity of an initiation factor will depend on experiments with an in vitro system synthesizing viral protein. Two types of experiments with such systems have been described, but one uses E. coli ribosomes and initiation factors (Rekosh, Lodish and Baltimore, 1970), and the other uses viral polyribosomes initiated in vivo (Roumiantzeff, Summers and Maizel, 1971). Thus neither
experiment tests the initiation of translation in vitro using mammalian cell ribosomes with or without initiation factors. Some experiments described in this thesis [sections 3.3(e) and 3.4(c)], as well as other experiments in this laboratory, have shown the difficulty of handling VP0, VP1 and VP3 preparations in vitro owing to their stickiness and readiness to aggregate.

(b) Complexes of viral ribonucleoprotein and ribosomes

The detection on caesium chloride gradients of species with the densities expected of complexes of ribosomes with vRNP, and carrying VP0, VP1 and VP3, is also consistent with the hypothesis that these viral proteins play a part in the initiation of protein synthesis. However it was not shown that these proteins were only at, or near, the 5' end of the vRNP, or were also attached to the small subunits in the polyribosomes.

The amino acid label associated with the ribosomal material did not represent nascent protein, as it consisted of three discrete and small proteins (<35,000 daltons) of reproducible size, rather than material of a spectrum of sizes extending to that of NCVP 1 (approximately 105,000 daltons). The absence of NCVP 2 and other viral protein indicated that the attachment of VP0, VP1 and VP3 was specific. It is not known whether these particular VP0, VP1 and VP3 molecules had existed free in the cytoplasm and rejoined as an initiation factor, or whether
a small proportion of the NCVP 1 molecules had adhered to
and were subsequently cleaved on the ribosome that produced
them.

The unequivocal determination of the existence
and density of a complex of [VP0, VP1, VP3]-vRNP-small
ribosomal subunit or a complex of [VP0, VP1, VP3]-vRNP-
ribosome must await the isolation of VP0, VP1 and VP3 in a
form suitable for use in synthesizing these postulated
polyribosome precursors in vitro.

Nevertheless, Figure 48 has been drawn with a
particle comprising VP0, VP1 and VP3 at the 5' end of viral
RNA in order to simplify interpretations. The density of
viral polyribosomes has previously been reported as 1.54
g/cc (Huang and Baltimore, 1970), 1.51 - 1.53 g/cc (Miller,
1972) and 1.52 g/cc (Fenwick and Wall, 1972). As depicted
in the diagram, the density of a polysome is expected to
increase with the ribosome loading. Treatment with EDTA of
viral polysomes labelled with \(^3\)H]uridine after infection
(J-M of Figure 48) led to the release of material with the
density of vRNP (Figure 33C). Following treatment with
EDTA of viral polysomes carrying labelled viral protein
(C-E of Figure 48), species with the densities of vRNP and
the small ribosomal subunit (30S in EDTA) were detected
(Figure 27B), suggesting the proximity of VP0, VP1 and VP3
to both the subunit and viral RNA in the original structure.

It is apparent on comparing Figures 25A and 33B
that the proportion of protein label in the 1.49 - 1.51 g/cc
and 1.51 - 1.54 g/cc regions was about equal, whereas the
majority of the uridine label was in the 1.51 - 1.54 g/cc region. This means that the ratio of VP0, VP1 and VP3 to vRNP was less in polyribosomes of higher density, i.e. the number of VP0, VP1 and VP3 molecules per ribosome was lower in larger polyribosomes. If this quantitative distribution could be confirmed in further experiments, some interesting inferences would be possible concerning the release of VP0, VP1 and VP3 following the initiation of protein synthesis.

(c) The effect of guanidine on ribosomal complexes

The main feature of the cytoplasmic extracts from cells treated with guanidine was the lack of viral protein on the 45S subunit and on the ribosomal-vRNP complexes, revealing the presence of vRNP that probably carried VP0, VP1 and VP3.

An explanation of these observations is possible if it is proposed that guanidine acts to increase (i) the affinity of VP0, VP1 and VP3 for the 5' end of viral RNA and also (ii) the tendency of VP0, VP1 and VP3 to aggregate into empty capsids. Action (i) would lead to prevention of the release of completed complementary RNA, resulting in the guanidon [section 3.4(a)]. Complementary RNA would continue to be synthesized, but would not be available to make viral RNA, accordingly reducing the number of viral polyribosome precursors. Both effects are likely to reduce the number of [VP0, VP1, VP3] particles free in the cell and therefore to shift the equilibrium, [VP0, VP1, VP3] + 45S subunit = [VP0, VP1, VP3] 45S subunit further to the left. Thus, fewer subunits would be
expected to carry labelled viral proteins, as was found to be the case, and the rate of viral protein synthesis would be expected to decay. It has been reported (Caliguiri and Tamm, 1968b; Baltimore, 1969) that the rate of protein synthesis is reduced by 30 - 50% one-half hour after the addition of the drug.

(d) The replication complex and guanidon

The structural proteins VP0, VPI and VP3 were also found in the guanidon, and in the replication complex of high sedimentation constant from normal infected cells. Their presence in the replication complex was also reported by Caliguiri and Mosser (1971), although their preparations were derived by deoxycholate treatment of a smooth membrane fraction of infected cells, and sedimented heterogeneously between 75S and 265S. They did not obtain a gel pattern of VP0, VPI and VP3 which was as free of high molecular weight contaminants as that given in Figure 43.

The results give no information on whether VP0, VPI and VP3 were present as a particle consisting of one molecule each of these polypeptides, or whether they were attached to the 5' end of viral RNA.

(e) The polypeptide composition of empty capsids and virions

The similarity in the molecular weights of Mahoney and ts+ VP3, together with the difference in size of VPI for the two strains and the small proportion of this polypeptide in ts+ virions (Figure 8) have been
already reported (Cooper, Summers and Maizel, 1970). However, the fact that VP0 of both strains is the same size has not been previously established. The gel patterns of empty capsids often revealed minor components between the major peaks of VP2 and VP3 (e.g. Figures 8 and 9), a finding that further indicates the variable nature of the cleavage of poliovirus protein (Cooper, Summers and Maizel, 1970; Cooper and Bennett, 1973; Vanden Berghe and Boeyé, 1972).

3.7 Summary

(i) The structural polypeptides VP0, VP1 and VP3 were detected on 45S ribosomal subunits, in species with the densities of ribosome-viral ribonucleoprotein complexes (1.50-1.54 g/cc), on viral ribonucleoprotein and in the replication complex of poliovirus-infected cells. The experiments involved gel electrophoresis and isopycnic centrifugation in caesium chloride after fixation of sucrose gradient fractions.

(ii) Attempts by disruption of ribonucleoprotein complexes to obtain structures with the expected sedimentation constant of free particles comprising one molecule each of VP0, VP1 and VP3 were unsuccessful except in the case of 45S ribosomal subunits. These failures probably resulted from a strong tendency of such particles to aggregate or to adhere to surfaces.

(iii) Ribosomal protein synthesis in cells infected with poliovirus was inhibited at the same rate as total protein
synthesis. With one possible exception, no evidence for selectivity in the inhibition of synthesis of host ribosomal proteins was obtained.

(iv) The sedimentation coefficient of empty capsids relative to ribosomes, and the molecular weights of their constituent polypeptides were found to be lower than the generally accepted values. Empty capsids of both the Mahoney and ts+ strains sedimented at about 65S. The molecular weights of VP0 and of VP3 in both strains were identical at 34,000 and 22,000 daltons respectively. VP1 from the Mahoney strain had a molecular weight of 30,000 daltons; VP1 from ts+ was 28,000 daltons.
# DNA-DEPENDENT RNA POLYMERASE ACTIVITY IN POLIOVIRUS-INFECTED CELLS

## 4.1 Introduction

## 4.2 Poliovirus-Induced Inhibition of RNA Polymerase Activity

## 4.3 DNA-Associated Proteins

## 4.4 Discussion

(a) RNA polymerase  
(b) DNA-associated proteins

## 4.5 Summary

<table>
<thead>
<tr>
<th>Source</th>
<th>Aggregate enzyme</th>
<th>Inhibition</th>
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<td>Uninfected cells</td>
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<td>246</td>
</tr>
<tr>
<td>Infected cells</td>
<td>4.9 mg RNAse</td>
<td>244</td>
</tr>
</tbody>
</table>

Cells 4.9 hr after infection

| Expt. 2 |                |            |
| Uninfected cells | 5 mg RNAse | 246 |
| Infected cells | 4.9 mg RNAse | 244 |

Cells 4.9 hr after infection

Table 4. Aggregate enzyme DNA-dependent RNA polymerase activity. Assay mixture (Holland, 1964) in 0.1 ml: HEPES-SCN, pH 5.5 50 mM, MgCl₂, 1.5 mM, dATP, dGTP, dCTP, dUTP each 0.1 mM, ETP 0.01 M, 33 A₂₆₀ units RNA, 0.015 μg template; "..."
<table>
<thead>
<tr>
<th>Source of aggregate enzyme</th>
<th>Addition</th>
<th>p moles [³H]CTP incorporated/30 min/mg protein</th>
<th>% inhibition</th>
</tr>
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<td>Expt. 1</td>
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<td>Uninfected cells</td>
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<td>Uninfected cells</td>
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<tr>
<td>Cells 4.5 hrs after infection</td>
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<td>20</td>
</tr>
</tbody>
</table>

Table 4. Aggregate enzyme DNA-dependent RNA polymerase activity. Assay mixture (Holland, 1964) in 0.5 ml; Tris-HCl pH 8.0 50 µmoles, MnCl₂ 1.5 µmoles, 2-mercaptoethanol 5 µmoles, 0.05 ml (NH₄)₂ SO₄ saturated at 4°C, brought to pH 8 with NH₄OH. UTP, GTP, ATP, each 0.4 µmole, CTP 0.04 µmole, [³H]CTP 0.015 µmole, approx. 300 µg protein.
4.1 Introduction

The aim of the work described in this chapter was to determine, if possible, the identity of the virus-coded protein present in infected cells responsible for the inhibition of host RNA synthesis. The first step was to confirm the inhibition of the DNA-dependent RNA polymerase activity measured at high ionic strength in aggregate enzyme preparations. This was extended to assaying polymerase activity at high and low ionic strengths in isolated nuclei.

The next step was to analyse by gel electrophoresis the polypeptides of aggregate enzyme for the presence of new proteins following infection. As aggregate enzyme is a mixture of polymerase and template, it was possible that this approach would lead to the detection of newly synthesized polypeptides affecting template and/or polymerase activities.

4.2 Poliovirus-Induced Inhibition of RNA Polymerase Activity

The activity of aggregate enzyme assayed at high ionic strength is shown in Table 4. It was inhibited by actinomycin D and deoxyribonuclease as expected for DNA-dependent RNA polymerase. The variation in the inhibition caused by virus infection has also been noted in Mengovirus-infected cells (Baltimore and Franklin, 1962), and may be due to experimental inconsistencies or to differences in the rate of host cell macromolecular synthesis at the time
Table 5. RNA polymerase activity of isolated nuclei from uninfected cells (CON) and cells 3 hrs after infection (INF). Assay mixture as for Table 4.
of infection. The cells in these experiments were not synchronized. However, the reduction of activity in U cells by poliovirus infection justified the investigation of aggregate enzyme by polyacrylamide gel electrophoresis. Since large quantities of cells were needed to prepare aggregate enzyme, further assays were carried out on isolated nuclei, and the cytoplasm from infected cells was tested for inhibitory factors. Two experiments are shown in Table 5. In both experiments the nuclei from infected cells had approximately 50% of the activity of those from uninfected cells. In the second experiment, the addition of cytoplasmic extract from infected cells to uninfected cell nuclei apparently inhibited the polymerase activity. The reverse was true in the first experiment. Because of the inconsistencies in these and other similar experiments, no attempts were made to purify inhibitory factors from the infected cell cytoplasm.

When this work was being done, the first reports on the presence of two different DNA-dependent RNA polymerase activities in mammalian cell nuclei were published (Novello and Stirpe, 1970; Goldberg, 1970; Goldberg and Moon, 1970). Polymerase 1 (or A) was assayed at low ionic strength in the presence of Mg\(^{++}\) whereas polymerase 2 (or B) was assayed at high ionic strength with Mn\(^{++}\) as in Tables 4 and 5. Accordingly, nuclei from uninfected and infected cells were assayed in these new incubation mixtures with and without 1 µg of α-amanitin, which was reported to
<table>
<thead>
<tr>
<th></th>
<th>Nuclei (stored at -60°C)</th>
<th>p moles $[^3]H$GTP incorporated/15 min/mg protein</th>
<th>% Inhibition</th>
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</thead>
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<td>INF</td>
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<tr>
<td></td>
<td>INF</td>
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<tr>
<td>Polymerase 2 in presence of 1 μg α-amanitin</td>
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<td></td>
</tr>
<tr>
<td></td>
<td>INF</td>
<td>26</td>
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</tr>
</tbody>
</table>

Table 6. RNA polymerase activity of isolated nuclei assayed at high and low ionic strength.

Assay mixtures: (Novello and Stirpe, 1970). Total volume 0.5 ml.

**Polymerase 1.**
50 μmoles Tris-HCl pH 8.0, 2 μmoles MgCl$_2$, 7 μmoles 2-mercaptoethanol, 3 μmoles NaF, 0.3 μmole CTP, ATP, UTP, 0.017 μmole GTP, 0.003 μmole $[^3]H$GTP, approx. 200 μg protein.

**Polymerase 2.**
50 μmoles Tris-HCl pH 8.0, 2 μmoles MnCl$_2$, 0.14 mmole (NH$_4$)$_2$SO$_4$, 0.9 μmole ATP, CTP, UTP, 0.051 μmole GTP, 0.009 μmole $[^3]H$GTP, approx. 200 μg protein. CON/INF as in Table 5.
Figure 49 Co-electrophoresis with $^{125}$I-labelled standard proteins of portions of aggregate enzyme preparations from cells treated as described in the text. The uninfected cells were labelled for 16 hr, and the infected cells from 0.5-2.5 hr after reversal of guanidine inhibition. Aggregate enzyme was prepared from $4 \times 10^8$ cells as described in section 2.8(b).
abolish polymerase 2 activity but to have no effect on polymerase 1 (Novello and Stirpe, 1970; Stirpe and Fiume, 1967). The data in Table 6 confirmed the inhibition of polymerase 2 by infection, and also showed that polymerase 1 activity was reduced. When polymerase 1 was assayed in the presence of α-amanitin there was a slight drop in activity which could have been due to the removal of background polymerase 2 activity, or because the nuclei used for the α-amanitin assays were stored four weeks longer. The polymerase 2 activity of the preparations assayed in the presence of α-amanitin was reduced by 93% to a level comparable to the total polymerase 1 activity.

4.3 DNA-Associated Proteins

Cells were infected with the Mahoney strain of virus, incubated with actinomycin D (0.3 μg/ml) and guanidine carbonate (200 μg/ml) in growth medium for 1.5 hours, washed free of the drugs, and then incubated a further 30 minutes in fresh medium lacking amino acids. [3H]leucine was added to 8 μc/ml, and after 2 hours' incubation the aggregate enzyme was prepared for electrophoresis. Figure 49 shows the gel pattern of the preparation from infected cells in comparison with that of a similar extract from uninfected cells which were labelled for 16 hours at 5 x 10^5 cells/ml with 0.25 μc/ml of [3H]leucine. There were no clear differences between the infected and uninfected cells, except in the high molecular weight region. However it was possible that most of an inhibitory
Figure 50  Co-electrophoresis with $^{125}$I-labelled standard proteins of aggregate enzyme preparations from cells treated as described in the text. The cells were labelled immediately after infection or "dummy" infection. Aggregate enzyme was prepared from $4 \times 10^8$ cells as in section 2.8(b).
Figure 51  Stained gels of (A) Tobacco Mosaic Virus protein (TMV); (B) cytochrome c; (C) TMV protein, cytochrome c, and the same preparation from infected cells used in Figure 49 co-electrophoresed.
protein was made in the interval between infection at 0°C and the addition of label, since the input viral RNA was almost certainly translated during this time.

Accordingly, cells were infected at 0°C and treated for 30 minutes at 37°C with actinomycin D (0.3 µg/ml) and cycloheximide at 200 µg/ml to stop all protein synthesis. The cells were washed and suspended immediately in medium lacking carrier leucine but with 2.2 µc/ml of [3H]leucine. After 2.5 hours' incubation the aggregate enzyme was prepared. The gel pattern of infected cells is shown in Figure 50A. The uninfected cells used for Figure 50B were treated the same way, except that ordinary medium replaced virus suspension in the first step. Again there were no obvious differences between uninfected and infected cells.

Figure 51C is a stained gel of the infected preparation of Figure 49A co-electrophoresed with tobacco mosaic virus protein (17,500 daltons) and cytochrome c (12,400 daltons). Excluding the possibility that bands were obscured by the marker proteins, especially cytochrome c, there were three major proteins. Using cytochrome c and TMV proteins as standards, the calculated molecular weights of these polypeptides were approximately 16,000, 15,500 and 14,000 daltons. The latter two probably corresponded to the two major peaks in Figures 49 and 50.

The next experiment was done for two purposes. The first was to test the hypothesis that the change in aggregate enzyme activity after infection was a consequence of the loss of host-coded protein, and the second was to quantitate the
inhibition of the synthesis of the DNA-associated proteins. 8 x 10^7 cells were first labelled for 2.5 hours with 6 μc of 14C-labelled Reconstituted Protein Hydrolysate in Eagle's medium lacking amino acids. They were then resuspended in identical medium without label and incubated a further 30 minutes. One half of the cells were infected at 0°C with ts virus, the other half were simply stirred in growth medium at 0°C. Thereafter both portions of cells were treated in the same way. Following the infection procedure, the cells were incubated for two hours in actinomycin D (0.3 μg/ml) and guanidine carbonate (200 μg/ml), washed with PBS, and resuspended in Eagle's medium lacking lysine. After 2.5 hours' incubation, [3H]lysine was added to 5 μc/ml and the incubation continued for a further 75 minutes.

At the end of this period the aggregate enzyme from each culture was extracted, with care being taken at each preparative step to reduce the loss of material to an absolute minimum. The final samples were prepared for electrophoresis and a portion of each counted in the scintillation counter. The 14C-radioactivity recovered in the infected and uninfected preparations was 4,100 and 4,000 cpm respectively. The corresponding figures for the 3H-radioactivity recovered were 6,700 and 34,000. Thus there was no apparent loss of prelabelled protein from the aggregate enzyme of infected cells, but the synthesis of DNA-associated proteins was inhibited by approximately 80%
after infection. The gel patterns of these samples (not shown) were similar to those of Figures 49 and 50, with no new proteins detectable after infection, and the same proteins labelled before and after infection.

4.4 Discussion

(a) RNA polymerase

The inhibition of cellular DNA-dependent RNA polymerase as a result of infection by poliovirus was reported some time ago (Holland, 1962). However, there have been conflicting reports on the ability of cytoplasmic extracts from infected cells to inhibit RNA polymerase activity in isolated nuclei (Holland, 1962; Balandin et al., 1965). The results in Table 5 suggested that any such inhibition by cytoplasmic extracts from infected U cells was insufficiently large or consistent to be amenable to further study.

The reduction in activity of both polymerase 1 and 2 (Table 6) by infection indicated that there was no marked selectivity in the inhibition of cellular RNA synthesis. The two enzyme forms have different locations in the nucleus and synthesize different classes of RNA (Goldberg and Moon, 1970).

(b) DNA-associated proteins

One of the surprising findings was the apparent resistance of the synthesis of the DNA-associated proteins to inhibition following infection. In the last experiment described in section 4.3, the cells were labelled
relatively late after reversal of guanidine inhibition (2.5 - 3.75 hours), at a time when a gel of the cytoplasm shows exclusively viral proteins, and yet the synthesis of DNA-associated protein was continuing at 20% of the rate in uninfected cells. Sokol, Cox, Dinka and Ackermann (1965) found that the synthesis of histones in infected cells was stimulated during the first two hours after poliovirus infection, but depressed by the third hour.

The most obvious peaks of low molecular weight in Figures 49-51 almost certainly corresponded to histones, as the five main histone fractions lie in the molecular weight range 10,000 - 25,000 daltons (Butler, Johns and Phillips, 1968; Robbins and Borun, 1967). The non-histone proteins vary in molecular weight from about 5,000 - 100,000 daltons (Elgin and Bonner, 1970) but are mostly larger than the histone proteins (MacGillivray, Carroll and Paul, 1971). Both histone and non-histone chromosomal proteins have been advocated as being important in the regulation of DNA transcription (see review by Stellwagen and Cole, 1969).

No significant qualitative differences in the chromosomal proteins made before and after poliovirus infection were discovered in this work, neither was any new viral protein that could account for the inhibition of DNA-dependent RNA polymerase activity found attached to the DNA. Quantitative calculations were not possible from the gels. However, it could not be foreseen that the synthesis of the chromosomal proteins would be so
refractory to inhibition after infection, and that minor changes would be difficult to detect among the host proteins still being synthesized. It is possible that a minor viral protein was present in the preparations, or that a viral protein had combined with and removed RNA polymerase core or associated factors, but if so, these events remained undetected because of the insensitivity of the techniques used.

4.5 Summary

The infection of cells with poliovirus decreased the activity of DNA-dependent RNA polymerase assayed at high ionic strength in aggregate enzyme, and assayed at both high and low ionic strength in preparations of nuclei. The radioactive polypeptides in aggregate enzyme after the labelling of infected cells were examined by gel electrophoresis and compared with those of uninfected cells. No new viral polypeptides or major changes in the relative amounts of host-coded proteins were detected.
CHAPTER FIVE
SMALL PLAQUE AND COLD SENSITIVE MUTANTS OF POLIOVIRUS

5.1 Introduction

5.2 The Isolation and Preliminary Characterization of the Mutants

5.3 Discussion and Summary

<table>
<thead>
<tr>
<th>5-Fluorouracil in growth medium</th>
<th>Temperature (°C)</th>
<th>E.O.P.</th>
<th>E.O.P.</th>
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<tr>
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<td>4 nd</td>
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<tr>
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<td>39.5</td>
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Table 7. The effect of growth at 37.2°C in the presence of 5-fluorouracil on the plating efficiency of the ED strain of virus at three temperatures. The plaque assay bottles at 37.2°C and 39.3°C were stained at 64 hrs; the bottles at 34.0°C were stained at 80 hrs.
### 5-Fluorouracil in growth medium

<table>
<thead>
<tr>
<th>Temperature °C</th>
<th>Pfu/ml x 10^-6</th>
<th>Yield compared with no 5-FU at 37.2°C</th>
<th>E.O.P. at 39.5°C compared with 37.2°C</th>
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<tr>
<td>0</td>
<td>12.0</td>
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</tr>
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<td>37.2</td>
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<td>1 mM</td>
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<td>39.5</td>
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Table 7. The effect of growth at 37.2°C in the presence of 5-fluorouracil on the plating efficiencies of the ts⁺ strain of virus at three temperatures. The plaque assay bottles at 37.2°C, 39.5°C were stained at 64 hrs; the bottles at 34.0°C were stained at 89 hrs.
5.1 Introduction

This final chapter of the experimental section of this thesis describes preliminary work on the isolation of small plaque and cold sensitive mutants of poliovirus. Mutants were isolated from a virus stock of the ts+ strain mutagenized by growth in 5-fluorouracil, but were not rigorously characterized by biochemical and genetic techniques.

5.2 The Isolation and Preliminary Characterization of the Mutants

Monolayers of 5 x 10^6 cells in Kimax bottles were inoculated with 8 x 10^7 pfu of the ts+ strain of virus. After one hour's absorption, the cells were washed with PBS and the cultures incubated at 37.2°C for 40 hours in growth medium containing 5-fluorouracil (5-FU). The 40 hour yield was determined by plaque assay at three temperatures (Table 7). This experiment was essentially that described by Cooper (1964b) and the findings were similar. (a) 5-FU inhibited the growth of poliovirus. (b) Increasing the 5-FU concentration decreased the plating efficiency at 39.5°C. (c) The efficiency of plating at 34°C was scarcely affected by increasing the 5-FU concentration. The last result suggested that cold sensitive mutants would be far less frequent than temperature (heat) sensitive mutants in the mutagenized stocks.

Virus grown in the presence of 4 mM 5-FU was assayed on 4" Petri plates, incubating them for 2 days at
Figure 52  A comparison of the plaque size of ts$^+$ and sp-14. The bottles were incubated at 37.2°C for 3 days before staining.
Figure 53. The growth at 33.5°C and 37.2°C of cs-75 compared with that of ts+. The bottles incubated at 37.2°C were stained after 3 days. Those incubated at 33.5°C were stained after 4 days.
$\text{ts}^+, 10^{-5}, 37.2^\circ\text{C}$

$\text{cs-75}, 10^{-3}, 37.2^\circ\text{C}$

$\text{ts}^+, 10^{-5}, 33.5^\circ\text{C}$

$\text{cs-75}, 10^{-3}, 33.5^\circ\text{C}$
Figure 54  The growth at 33.5°C and 37.2°C of cs-477 compared with that of ts+. Incubation times as in Figure 53.
\( ts^+, 10^{-5}, 37.2^\circ C \)

\( cs-477, 10^{-4}, 37.2^\circ C \)

\( ts^+, 10^{-4}, 33.5^\circ C \)

\( cs-477, 10^{-3}, 33.5^\circ C \)
<table>
<thead>
<tr>
<th>Virus Strain</th>
<th>Plating Efficiency at 33.5°C compared with that at 37.2°C</th>
<th>Plaque diameter (mm) at 37.2°C</th>
<th>Plaque diameter (mm) at 33.5°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>ts⁺</td>
<td>0.8 - 1.0</td>
<td>3-4</td>
<td>2.5 - 4</td>
</tr>
<tr>
<td>sp-14</td>
<td>- a</td>
<td>0.5 - 1</td>
<td>- a</td>
</tr>
<tr>
<td>cs-75</td>
<td>0.01</td>
<td>2-3</td>
<td>&lt;1</td>
</tr>
<tr>
<td>cs-167</td>
<td>0.02</td>
<td>3-4</td>
<td>2.5 - 4</td>
</tr>
<tr>
<td>cs-477</td>
<td>0.1</td>
<td>2-3</td>
<td>1 - 1.5</td>
</tr>
</tbody>
</table>

Table 8. The properties of the mutants summarized in this table were based on the results of three plaque assays in aspirin bottles (section 2.3). Bottles incubated at 37.2°C were stained at 64 hrs; those incubated at 33.5°C were stained at 89 hrs.

* Plaques did not appear within 4 days at 33.5°C.
Table 9. The growth of small plaque and cold sensitive mutants at 37.2°C and 33.5°C. 10³ cells in suspension cultures were infected at 0°C at an input multiplicity of 0.1 pfu/cell, and the total pfu produced after 8 hours determined by plaque assays at 37.2°C.
37.2°C, then for 2 days at 34.0°C before staining with the ethanol based INT spray. Small plaques were picked, grown up in tube cultures, and spot tested (Cooper, 1964b) in 4" Petri plates at the two temperatures. Promising isolates were more accurately tested by plaque assay of serial dilutions in aspirin bottles, recloned and then grown up in Kimax bottles. Final titres were $4 \times 10^6$ pfu/ml.

The small plaque mutant sp-14 is illustrated in Figure 52; the cold sensitive mutants cs-75 and cs-477 are shown in Figures 53 and 54 respectively. In the particular assay photographed for these two figures the efficiency of plating of $ts^+$ at 33.5°C compared with 37.2°C was approximately 0.65; on most occasions it was 0.8 - 1.0. The efficiencies of plating of cs-75 and cs-477 were 0.02 and 0.12 respectively. Not shown is cs-167, a mutant that gave wild-type plaques at 37.2°C and none at 33.5°C except some of wild-type character at a frequency of 2% of the plaques at 37.2°C. The properties of the mutants are summarized in Table 8. Cystine, known to affect the plaquing ability of some $ts$ mutants (McCahon and Cooper, 1970), had no effect on sp-14 and cs-167 when used at 0.20 mM and 0.02 mM in the agar medium.

One experiment was done to determine the number of pfu produced per infected cell for each of the mutants and $ts^+$ (Table 9). Since the input multiplicity was less than 0.1 pfu/cell, more than 95% of the infected cells adsorbed only one pfu. Hence the figures in the last two
columns of Table 9 essentially give the number of pfu produced per infected cell. None of the mutants showed marked defects in their ability to adsorb to cells at 0°C, but none produced as many pfu/cell as \( t_s^+ \) at either temperature. The yields of \( t_s^+ \) grown at 33.5°C and 37.2°C were similar, but the yield at the lower temperature was depressed for all the mutants.

5.3 Discussion and Summary

One small plaque and three cold sensitive mutants were isolated. \( cs-75 \) and especially \( cs-477 \) showed too much leak at the non-permissive temperature for genetic mapping to be considered. The stock of \( cs-167 \) prepared contained 2% wild-type virus, indicating a high reversion rate for this mutant.

The physiological characterization of these mutants was not taken sufficiently far for the biochemical lesions determining their defective behaviour at low temperature to be discovered. Thus it is not yet known whether the mutants are defective in regulatory functions.
The more significant findings of this thesis are those described in Chapter Three. Although the work on the inhibition of host RNA synthesis (Chapter Four) yielded further information about this event in infected cells, no promising approaches for future experiments were suggested until purified viral and cellular RNA polymerases have been physically characterized. Such purification will enable a comparison of their components, in particular a comparison of the composition of the cellular polymerase before and after infection. The study of the small plaque and cold sensitive mutants (Chapter Five) did not advance sufficiently for significant new findings to be made.

The rest of this chapter is devoted to considering the possible implications of the presence of VP0, VP1 and VP3 in ribosomal and replication complexes of infected cells. The results are discussed in terms of the equestron model (Cooper et al., 1973). The observations suggesting the action of a regulator during the poliovirus growth cycle were outlined in section 1.1 (e). The essential property of the regulator proposed in the model is that it should be multifunctional. It should have an affinity for the 45S subunits that both decreases the subunits' interaction with host messenger RNA and increases their interaction with viral RNA, and have an affinity for viral RNA that both increases viral
RNA interaction with 45S subunits and affects viral RNA replication. The regulator can be expected to be labile so that its affinity for viral RNA does not prevent the formation of virions.

The reasons for suggesting that the regulator might be a particle composed of VP0, VP1 and VP3 are as follows. (i) The native 45S subunits of infected cells carry VP0, VP1 and VP3, and a radioactive particle of 6S can be obtained from subunits carrying these labelled polypeptides [section 3.3(a)]. (ii) Ribosomal structures, tentatively identified to be polysome precursors, also carry these three polypeptides [section 3.3(b)], which after disruption with EDTA are found in part on vRNP. (iii) VP0, VP1 and VP3 are found in the replication complex and guanidin of infected cells (section 3.4(b); Caliguiri and Mosser, 1971). (iv) ts mutants that are defective in the repression of host protein synthesis at the restrictive temperature map in the structural protein region of the poliovirus genome (Steiner-Pryor and Cooper, 1973). (v) Mixed infection of cells with certain ts mutants and ts+ at the restrictive temperature suppresses RNA synthesis of ts+; these interfering mutants also map in the structural protein region (Cooper, Wentworth and McCahon, 1970). (vi) VP0 is cleaved to VP2 and VP4 (Jacobson et al., 1970; Summers et al., 1971), and hence a regulator composed of VP0, VP1 and VP3 would have the lability to allow virus maturation. (vii) A regulator
that is also structural protein, overcomes the problem of
the lack of "genetic room" [section 1.1(a)] for protein
having only regulatory functions.

Each of the above facts is consistent with, but
of course does not prove, the hypothesis that a particle of
one molecule each of VP0, VP1 and VP3 constitutes the
proposed regulator (the equestron) and has affinities for
the 45S ribosomal subunit and for the 5' end of viral RNA.
The proposed regulatory system based upon the equestron
is outlined below in terms of current ideas of viral
replication (Baltimore, 1969) and of ribosome function
(Lucas-Lenard and Lipmann, 1971).

(i) Initiation of infection (0 - 2 hours)

The first achievement of a single invading viral
RNA molecule must be to join a ribosome and begin
translation. Because structural protein is nearest the 5'
end [see section 1.1(a)], the first functioning gene
product should be equestron proteins, available within five
to ten minutes to form reversible complexes with native
45S subunits, viral RNA or both. This link between the
45S subunit and the viral RNA increases its rate of
translation. More equestrons are made and the 45S -
equestron complexes block the initiation of protein
synthesis on host messenger RNA. If the equestron's
affinity for the 45S subunit is much higher than its
affinity for the 5' end of viral RNA, equestrons will rarely
exist on viral RNA until the 45S subunits approach
saturation. From the decrease in host protein synthesis this should be two to three hours after infection, depending on multiplicity (Penman and Summers, 1965).

Poliovirus complementary RNA is transcribed from incoming viral RNA, and progeny viral RNA from complementary RNA, by two different virus-coded replicases or replicase factors (Cooper, Stanček and Summers, 1970). As the last poliovirus genes translated (at the 3' terminus) are probably those coding for the replicases (Cooper et al., 1971), the protein that is to transcribe the first invading RNA strand is released close to the site where it commences the replication process. Hence the first round of translation is probably followed rapidly by a round of transcription. During the first two hours after infection there will be few equestrons on viral RNA and thus not much restriction on the function of the replicases. Viral RNA production will outstrip viral protein production initially.

(ii) Midcycle (2 - 3.5 hours)

The increasing pool of equestron - 45S subunits takes a growing proportion of viral RNA, which now attaches to ribosomes within minutes (Huang and Baltimore, 1970). Thus the amount of template available for initiation of complementary RNA synthesis is reduced. During the viral RNA doubling time of fifteen minutes, each viral RNA strand in a polysome has sufficient time to make the required number of structural protein copies (60), since there are approximately 35 to 40 ribosomes per polysome (Rich et al.,
1963; Summers et al., 1967) and each takes about ten minutes to move the full length of a viral RNA molecule (Rekosh, 1972). Although the replicases are increasing sixty times as fast as viral RNA molecules, the doubling time is never shortened and so does not depend on replicase affinities.

(iii) Late RNA synthesis (3.5 - 4.5 hours)

Equestrons are increasing sixty times faster than viral RNA, and as the excess over those complexed with 45S subunits increases, they form reversible complexes with viral RNA. Consequently the time during which the 5' ends of viral RNA are free of attached equestrons decreases, and the completion and release of complementary RNA also decreases. Ultimately the 5' termini are never free of equestrons and the completion of complementary RNA stops. The kinetics of RNA synthesis change from exponential to linear (Baltimore, Girard and Darnell, 1966) at about 3.5 hours, and the cell makes use of those complementary RNA templates already completed.

(iv) Maturation

The virion protein VP2 is derived by cleavage from VP0 (Jacobson et al., 1970), and label added to infected cells takes at least thirty minutes to appear in VP2 (Summers and Maizel, 1968). A delay in the production of virion protein in the form necessary for maturation prevents viral RNA from being sequestered into virions before sufficient capsid material has been made, i.e. it
allows the template potential of the early viral RNA for further RNA or protein synthesis to be realized. However, the change from exponential to linear kinetics in the production of RNA and protein soon causes a drop in the equestron content of infected cells. This occurs because equestrons increase linearly but continue to decay exponentially by cleavage. The success of the equestron in bringing RNA and consequently protein synthesis to a linear rate, ultimately leads to its own disappearance with the maturation of structural units into virions.

Thus during the growth cycle, (i) - (iv) above, the equestron determines the timing of the cessation of host protein synthesis, the switch from exponential to linear RNA synthesis and finally virus maturation.

(v) The effect of guanidine

It is proposed that guanidine greatly increases the binding of the equestron by viral RNA (Cooper et al., 1973), presumably by a reversible conformational change in VP0 that also impairs its cleavage and encourages the formation of empty capsids (Jacobson and Baltimore, 1968b). Strong attachment of equestrons to the 5' ends of viral RNA can explain the blocking of new polysome recruitment (Huang and Baltimore, 1970), the stopping of maturation (Jacobson and Baltimore, 1968b), and the prevention of the completion and release of complementary RNA, leading to the guanidon (Baltimore, 1968), as equestrons on viral RNA aggregate and uncompleted complementary RNA accumulates.
The genetic evidence that guanidine resistance maps in the structural protein region of the poliovirus genome (Cooper, 1969), the fact that guanidine sensitivity is dominant in mixed infections of sensitive and resistance strains in the presence of guanidine (Pohjanpelto and Cooper, 1965; Cooper, Wentworth and McCahon, 1970) and the fact that guanidine has no effect on poliovirus replicase in vitro (Baltimore, Eggers, Franklin and Tamm, 1963), all support the proposal that guanidine mediates its effects through structural protein.

In conclusion, an attempt has been made in this final chapter to relate the ubiquitous distribution of VP0, VP1 and VP3 to a concept for regulation of poliovirus growth. The findings described in Chapter Three are consistent with this concept and provide a part of the evidence on which it is based. More experiments must be done in vitro with cell-free systems on the binding of viral proteins by viral RNA and ribosomes, and on systems synthesizing viral protein and RNA, before the postulated role of the equestron in regulating these processes can be properly tested. For the present, the equestron model provides a framework giving direction to further experimentation.
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