THE POLYPEPTIDES OF POLIOVIRUS AND THEIR SYNTHESIS

By

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STATEMENT

All experimental work reported in this thesis was done by myself.

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THESIS SUMMARY

The work described in this thesis represents an endeavour to gain a better understanding of aspects of poliovirus polypeptides and their synthesis. Autoradiography of dried acrylamide gel slices allowed the number of known poliovirus polypeptides to be considerably extended. This technique provided improved resolution allowing several minor components to be detected and differences in the polypeptide compositions of whole infected cells and cytoplasmic extracts of infected cells to be demonstrated. A more detailed examination of the extensive cleavage process known to occur during the synthesis of poliovirus polypeptides was achieved which confirmed previous observations that the cleavage specificity was the same throughout infection.

Screening of amino acid analogues for their ability to interfere with cleavage showed that, with one exception, only analogues with aromatic side chains caused significant alterations to polypeptide patterns. An unexpected result was that analogues of the dissimilar amino acids, arginine and phenylalanine, interfered with the cleavage process in an almost identical fashion. Also, three new compounds known to inhibit specifically certain proteolytic enzymes were shown to alter the specificity of the cleavage mechanism. Large precursor molecules induced in poliovirus-infected cells by different mechanisms were shown to accumulate in different ratios. Determination by gel electrophoresis of the size of the primary translation product of poliovirus RNA showed it to be 210,000 daltons. This finding implies that approximately 20% of the viral genome remains untranslated in vivo.
Electrophoresis of poliovirus polypeptides in acrylamide gels containing urea in addition to sodium dodecyl sulphate produced patterns different from those seen in the presence of detergent alone. The apparent molecular weights of some poliovirus polypeptides were shown to vary considerably depending on the composition of the gels used. These findings have important implications for the interpretation of analyses in dodecyl sulphate gels. The electrophoretic behaviour of the polypeptide VP3 was irregular and varied with the experimental conditions. This finding clarified previous conflicting results about the nature of this polypeptide.

Attempts were made to determine the initial steps in the cleavage of the primary translation product of poliovirus RNA. Tryptic peptide analyses allowed certain relationships among several polypeptides to be established. Radioactive samples eluted from gels in the regions of two prominent poliovirus polypeptides produced tryptic peptide maps of unexpected complexity. Interpretation of these results was that two polypeptide species may be present in both of these regions.

Attempts were made to label specifically in vivo the peptide involved in the initiation of translation of the poliovirus message. Low levels of radioactivity were incorporated into animal cells from exogenously-supplied N-formyl-\textsuperscript{35}S-methionine. However, analysis of cells labelled in this way showed that radioactivity was not positioned specifically at the N-termini of newly-synthesized polypeptides.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Section</th>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>STATEMENT</td>
<td>1</td>
</tr>
<tr>
<td>SUMMARY</td>
<td>11</td>
</tr>
<tr>
<td>CHAPTER ONE</td>
<td></td>
</tr>
<tr>
<td><strong>INTRODUCTION - A LITERATURE REVIEW</strong></td>
<td></td>
</tr>
<tr>
<td>1.1 The Strategy of Riboviruses</td>
<td>2</td>
</tr>
<tr>
<td>1.2 The Strategy of Poliovirus</td>
<td>5</td>
</tr>
<tr>
<td>1.3 The Analysis of Polypeptides in Poliovirus-infected Cells</td>
<td>11</td>
</tr>
<tr>
<td>1.4 The Synthesis of Poliovirus Polypeptides</td>
<td>14</td>
</tr>
<tr>
<td>1.5 Aspects of Polypeptide Synthesis and Processing in Eucaryotes</td>
<td>22</td>
</tr>
<tr>
<td>CHAPTER TWO</td>
<td></td>
</tr>
<tr>
<td><strong>METHODS AND MATERIALS</strong></td>
<td></td>
</tr>
<tr>
<td>2.1 Cell Cultures</td>
<td>30</td>
</tr>
<tr>
<td>2.2 Poliovirus Stocks</td>
<td>32</td>
</tr>
<tr>
<td>2.3 Preparation of Radioactive Virus and Empty Capsids</td>
<td>32</td>
</tr>
<tr>
<td>2.4 Polyacrylamide Gel Electrophoretic Analysis</td>
<td>33</td>
</tr>
<tr>
<td>2.5 Preparation of Cytoplasmic Extracts by Dounce Homogenization</td>
<td>36</td>
</tr>
<tr>
<td>2.6 Analysis of Poliovirus RNA in SDS-Sucrose Gradients</td>
<td>36</td>
</tr>
<tr>
<td>2.7 Preparation and Analysis of Tryptic Digests of Individual Polypeptides</td>
<td>37</td>
</tr>
<tr>
<td>2.8 Analysis of Amino Acids by Paper Chromatography</td>
<td>38</td>
</tr>
<tr>
<td>2.9 Preparation of N-formyl-$^{35}$S-methionine</td>
<td>39</td>
</tr>
<tr>
<td>2.10 Preparation of Transfer RNA from Cells</td>
<td>40</td>
</tr>
<tr>
<td>Section</td>
<td>Page</td>
</tr>
<tr>
<td>------------------------------------------------------------------------</td>
<td>------</td>
</tr>
<tr>
<td>2.11 Analysis of Ribonuclease Digests of Transfer RNA by Paper</td>
<td>40</td>
</tr>
<tr>
<td>Electrophoresis</td>
<td></td>
</tr>
<tr>
<td>2.12 Sources of Reagents</td>
<td>40</td>
</tr>
<tr>
<td>CHAPTER THREE</td>
<td></td>
</tr>
<tr>
<td>THE POLYPEPTIDES OF POLIOVIRUS-INFECTED CELLS</td>
<td></td>
</tr>
<tr>
<td>3.1 Introduction</td>
<td>45</td>
</tr>
<tr>
<td>3.2 The Polypeptides of Poliovirus Detected by Autoradiography</td>
<td>46</td>
</tr>
<tr>
<td>3.3 Proteolytic Cleavage Demonstrated by Pulse-Chase Experiments</td>
<td>51</td>
</tr>
<tr>
<td>3.4 Apparent Differences between the Polypeptide Compositions of Whole</td>
<td>58</td>
</tr>
<tr>
<td>Infected Cells and Cytoplasmic Extracts of Infected Cells</td>
<td></td>
</tr>
<tr>
<td>3.5 Discussion</td>
<td>60</td>
</tr>
<tr>
<td>3.6 Summary</td>
<td>64</td>
</tr>
<tr>
<td>CHAPTER FOUR</td>
<td></td>
</tr>
<tr>
<td>THE EFFECTS OF MODIFIED CULTURAL CONDITIONS ON THE</td>
<td></td>
</tr>
<tr>
<td>POLYPEPTIDES OF POLIOVIRUS-INFECTED CELLS</td>
<td></td>
</tr>
<tr>
<td>4.1 Introduction</td>
<td>66</td>
</tr>
<tr>
<td>4.2 The Screening of Various Amino Acid Analogues for Effects on</td>
<td>66</td>
</tr>
<tr>
<td>Proteolytic Cleavage</td>
<td></td>
</tr>
<tr>
<td>4.3 The Screening of Various Inhibitors of Proteolytic Enzymes for</td>
<td>72</td>
</tr>
<tr>
<td>Effects on Proteolytic Cleavage</td>
<td></td>
</tr>
<tr>
<td>4.4 The Effects of Temperature on Proteolytic Cleavage</td>
<td>74</td>
</tr>
<tr>
<td>4.5 A Comparison of the Very Large Poliovirus Polypeptides Induced by</td>
<td>76</td>
</tr>
<tr>
<td>Three Different Methods</td>
<td></td>
</tr>
<tr>
<td>4.6 The Molecular Weights of Poliovirus Polypeptides</td>
<td>78</td>
</tr>
</tbody>
</table>
CHAPTER FIVE

ANOMALOUS ELECTROPHORETIC BEHAVIOUR OF SOME POLIOVIRUS POLYPEPTIDES IN VARYING CONDITIONS OF SDS-GEL ELECTROPHORESIS

5.1 Introduction

5.2 The Effects of Urea on the Migration of Poliovirus Polypeptides

5.3 The Effects of Two Other Modifications on Electrophoretic Mobilities

5.4 Evidence that the VP3 Region is Comprised of One Principal Amino Acid Sequence

5.5 Discussion

5.6 Summary

CHAPTER SIX

INTER-RELATIONSHIPS AMONG SEVERAL LARGE POLIOVIRUS POLYPEPTIDES DEMONSTRATED BY PEPTIDE MAPPING

6.1 Introduction

6.2 Comparison of Tryptic Peptide Maps of Several Poliovirus Polypeptides

6.3 Discussion

6.4 Summary

CHAPTER SEVEN

ATTEMPTS TO LABEL IN VIVO THE INITIATION SITE OF TRANSLATION OF POLIOVIRUS RNA

7.1 Introduction

7.2 The Incorporation of N-formyl-\(^{35}\)S-methionine into Mammalian Cells

7.3 The Location on Transfer RNA of Radioactivity Derived from Exogenous N-formyl-\(^{35}\)S-methionine
CHAPTER ONE

INTRODUCTION - A LITERATURE REVIEW

1.1 The Strategy of Riboviruses
1.2 The Strategy of Poliovirus
   (a) The gene functions of poliovirus
   (b) The over-all growth cycle
   (c) Integration of the synthetic processes
1.3 The Analysis of Polypeptides in Poliovirus-infected Cells
   (a) The principles of SDS-gel electrophoresis
   (b) The detection of poliovirus polypeptides in acrylamide gels
1.4 The Synthesis of Poliovirus Polypeptides
   (a) The polypeptides of poliovirus-infected cells
   (b) The \textit{in vitro} synthesis of poliovirus polypeptides
   (c) The molecular weights of poliovirus polypeptides
1.5 Aspects of Polypeptide Synthesis and Processing in Eucaryotes
   (a) Initiation of translation
   (b) Post-translational cleavage of polypeptides
The sequence of events following the infection of cells with poliovirus has been extensively studied. Many of the processes are understood at a superficial level but large gaps still exist in the understanding of the mechanisms of synthesis, regulation and assembly. The experimental work described in this thesis attempts to clarify some aspects of the synthesis of poliovirus proteins. Chapter One is a review that relates the synthetic functions of poliovirus to other events in the infected cell, compares the mechanisms of protein synthesis and processing with those found in similar systems, and evaluates the methods used to analyse and detect them. Later experimental chapters describe advances made in the understanding of both the synthesis of poliovirus polypeptides and the methods used to detect them.

1.1 The Strategy of Riboviruses

The basic functions of all viruses are to infect their host cells, express their genetic information and to assemble the products into completed particles. A major differentiating factor between the deoxyriboviruses and the riboviruses is that in some of the latter, the genome acts as the message carrier and as such, is involved both in the replication and translation of the genetic information. The alternative class of riboviruses has its genetic coding in a form complementary to the functional messenger RNA.

Of those riboviruses in which the infecting genome may act as a messenger RNA, the best understood are the bacteriophages which have been recently reviewed by Hindley (1973). In summary, these phages have 3 cistrons which are translated independently in a regulated fashion. The cistron coding for the RNA polymerase activity (which is located at the
3' end of the genome) is translated first to increase the number of potential genomes, after which the cistrons coding for the maturation protein (5' end) and coat protein are translated. The control mechanisms for this regulation are remarkably sophisticated and do not depend on the polarity of the cistrons in the genome. The fact that this genetic message is punctuated for controlled translation reflects an ability of the host bacterial cell to initiate translation at more than one site in a polycistronic messenger RNA. This same molecule is also replicated during infection to produce a template from which progeny RNA may be transcribed.

Animal riboviruses which employ a similar strategy are the picornaviruses and possibly the arboviruses. In both of these groups the extracted RNA is infectious and so must participate directly in both translation and transcription. However, they use a different tactic because of the apparent inability of their host cells to terminate and reinitiate protein synthesis from a single, polycistronic RNA molecule (Jacobson & Baltimore, 1968b). Picornaviral RNA acts as a single messenger from which is translated a single large polypeptide which is later cleaved to produce smaller, functional proteins (Baltimore, 1969). Arboviruses are not yet clearly understood, but post-translational cleavage appears to have some role in protein synthesis (Burrell, Martin & Cooper, 1970; Schlesinger & Schlesinger, 1972).

The rhabdoviruses and paramyxoviruses represent a type of "negative strand" ribovirus in which the base sequence of the genome is complementary to the functional messenger RNA. In this case, punctuation is achieved by transcribing monocistronic messages from the infecting genome,
which are then individually translated (Huang, Baltimore & Stampfer, 1970; Bratt & Robinson, 1967). How replication of the genome is achieved to produce uninterrupted progeny molecules is not yet understood.

The strategy of the myxoviruses, which have a single-stranded, segmented genome, appears to be similar to that of the picornaviruses in terms of genome replication. The genetic content of each segment appears to be monocistronic (Skehel, 1972) and some evidence exists that they may function directly as messages (Siegert, Bauer & Hofschneider, 1973). Also segmented is the genome of the double-stranded reoviruses. This group transcribes a monocistronic messenger from each segment (Bellamy & Joklik, 1967), which in turn is translated into a single polypeptide of corresponding molecular weight (Smith, Zweerink & Joklik, 1969).

Uncertainty exists in the strategy of the only other major ribovirus group, the oncornaviruses. The genome of these viruses can be faithfully translated in cell-free systems (Siegert et al. 1972; Gielkens et al. 1973), but the mechanisms operative in vivo are unknown. The role of DNA transcription in the life cycle of these viruses is not yet certain.

The major ribovirus groups differ also with respect to the initial step in genome replication. All groups except the arboviruses and the picornaviruses carry in their virions polymerases capable of copying the infecting RNA molecules. It follows that these two groups must have a unique mode of regulation to allow the separate translation and transcription of a single infecting RNA molecule.
Post-translational cleavage plays a major part in the strategy of the picornaviruses. Although such cleavages have been found as well in a reovirus (Zweerink & Joklik, 1970), a myxovirus (Skehel, 1972), an arbovirus (Burrell et al. 1970) and an oncornavirus (Vogt & Eisenman, 1973) their role seems to be a minor one in the overall synthetic process.

In summary, the basic strategy of all the riboviruses is similar, but diversity occurs in the mode of presentation of messenger RNA to the host cell. The subsequent tactics of the mammalian riboviruses are further complicated by the inability of their host cells to translate polycistronic messages. Two features of the replication of poliovirus - the extensive cleavage process and a unique regulatory mechanism - therefore distinguish it (and other picornaviruses) from the other major groups of riboviruses. These two mechanisms are probably integrated and are considered more fully in following sections.

1.2 The Strategy of Poliovirus

The poliovirus genome is a single strand of RNA of molecular weight \(2.6 \times 10^6\) daltons (Granboulan & Girard, 1969; Tannock, Gibbs & Cooper, 1970) which has a potential coding capacity for approximately 2,500 amino acids or 270,000 daltons of protein. The genome functions as a single messenger RNA (Jacobson, Asso & Baltimore, 1970) and so is considerably larger than the average-sized messenger found in nature. As the gene products of the poliovirus genome are not translated individually, the classical genetic concept of "one gene, one polypeptide chain" has a different meaning for poliovirus.
(a) The gene functions of poliovirus

If a gene is defined as the smallest translatable unit of genetic material, then poliovirus has one gene only. However, mapping techniques have revealed three genetically separable activities (Cooper et al. 1971) which may be called primary gene functions, all of which are directly concerned with the synthesis of virion components. The genetic map of temperature sensitive mutants of poliovirus has been reviewed elsewhere (Cooper, 1969; Cooper et al. 1971). In summary, mutants defective in the viral coat protein occupy 48% of the genetic map and are found at the opposite end of the genome to those mutants defective in the synthesis of viral RNA. Two separate regions are found for the latter. At the restrictive temperature, mutants nearer the coat protein region synthesize double-stranded RNA only, while those towards the far end of the map synthesize neither double- nor single-stranded RNA (Cooper, Stancék & Summers, 1970; Cooper et al. 1971). These results suggested that two enzyme activities functioned in RNA replication; one synthesizing RNA complementary to the infecting strand and another synthesizing more virion RNA using the newly-made complementary RNA as a template. This proposal seemed consistent with models for the replication of poliovirus RNA (see Baltimore, 1969).

Experiments designed to specifically induce mutations at the 3' or 5' ends of poliovirus RNA, followed by genetic mapping, suggested that the polymerase functions mapped towards the 3' end and the coat protein mutants towards the 5' end (Cooper et al. 1971). Confirmation of this ordering was provided by experiments using the drug pacta-
mycin, which allowed the preferential labelling of poly-peptides translated from the 3' end of messenger RNA (Taber, Rekosh & Baltimore, 1971; Summers & Maizel, 1971). The relative sequence of three of the principal translation products was thus obtained, which showed that the precursor to the virion structural proteins (NCVP1) was translated from the region close to the 5' end of the molecule, while the large stable polypeptide (NCVP2) was translated from the region near the 3' end. Another stable polypeptide (NCVPX) seemed to be positioned between the other two.

The map region at the 3' end of the genome concerned with RNA synthesis has a much larger coding capacity than the region mapping towards the middle of the genome (Cooper et al. 1971). This corresponds with the observation that NCVP2 is about twice the size of NCVPX and so it is tempting to suggest polymerase functions for these two proteins (or their cleavage products or precursors). However, no information is yet available to identify the polymerase protein(s).

Other functions seen in poliovirus-infected cells are less-obviously related either to regions of the genome or to specific polypeptides. The inhibition of host macromolecular synthesis following infection is characteristic of picornaviruses (Martin & Kerr, 1968; Baltimore, 1969). With poliovirus, the decline in the rate of synthesis of host proteins precedes that of RNA, while the decline in DNA synthesis occurs later still. Gene expression is necessary for inhibition of host protein synthesis as it can be prevented by the use of p-fluorophenylalanine (Baltimore & Franklin, 1963), or by ultraviolet light-inactivated virus
(Penman & Summers, 1965). Similar experiments show that some virus-induced product is necessary for the inhibition of host RNA and DNA synthesis (Franklin & Baltimore, 1962). However, such inhibitory functions have not been shown conclusively for any specific poliovirus proteins, although Steiner-Pryor & Cooper (1973) suggest that a product of the structural protein gene may be the effector.

Recent studies on the activities of double-stranded RNA have suggested possible inhibitory roles for this species on host macromolecular synthesis. Ehrenfeld & Hunt (1971) showed that double-stranded RNA from several sources including poliovirus-infected cells could inhibit the initiation of protein synthesis in cell-free systems. Also, Cordell-Stewart & Taylor (1971) demonstrated the rapid cytotoxic effect of picornaviral double-stranded RNA on uninfected cells. Membranes and organelles were quickly damaged and effects were produced resembling the cytopathic effect seen late in picornaviral infections. It seems that double-stranded picornaviral RNA may have generalized inhibitory and toxic activities on the host cell accounting for the cytological changes seen late in the infection cycle. Hence, there is evidence that a product of a polymerase activity coded for by the poliovirus genome expresses secondary functions in the infected cell.

Similarly, the coat protein precursor (NCVPl) in a partially cleaved form may have other functions in limiting host protein synthesis, and regulating the synthetic activities of the infected cell (Cooper et al. 1973). This duplication of activities by the primary gene functions demonstrates genetic economy and suggests that further
searches for specific polypeptides having functions other than that for the replication of RNA may not be fruitful. The size of the structural protein gene relative to the scale of the genetic map (Cooper et al. 1971; Cooper & Bennett, 1973), and the difficulty of isolating mutants in novel functions suggests that all gene functions have probably been detected. If the polypeptides NCVP2 and NCVPX do represent the genetic activities concerned with RNA replication, then the sizes of these two polypeptides and the coat protein precursor are such that they can account satisfactorily for the whole of the translation product of poliovirus RNA (Cooper & Abraham, to be published). If this proposal is correct, it leads to a much simpler appreciation of the genetic make-up of poliovirus. Further support for the proposal is presented later in this thesis.

(b) The over-all growth cycle

A single step growth cycle of poliovirus occupies about 6 hours. The first 0.5 h of this period is concerned with the adsorption, penetration and uncoating of the infecting particle, and the initiation of the replicative sequences. The actual times at which the following processes occur vary slightly with the type of host cell and can be advanced by increasing the multiplicity of infection (Baltimore, Girard & Darnell, 1966).

The first observable change in the picornavirus-infected cell is a decrease in the rates of both host protein and RNA synthesis (Franklin & Baltimore, 1962; Holland, 1963). Both of these activities require some prior expression of the virus genome as they can be prevented by using amino acid analogues or ultraviolet light to inactivate the virus (Baltimore & Franklin, 1963; Penman & Summers, 1965). Such
viral products must be made within 1 h of infection but cannot be detected by present techniques. Although at least one cycle of translation of the infecting RNA must necessarily occur before transcription, both processes appear to proceed together, except that the synthesis of the bulk of the viral protein precedes that of the RNA by about one hour. The synthesis of virion RNA (which also acts as messenger RNA) increases exponentially from 1 to 3 h after infection, after which it increases linearly for a further 1 h (Baltimore et al. 1966). By about 3 h after infection, the host polyribosomes have been totally broken down and all subsequent protein synthesis uses the pool of newly-synthesized viral RNA. Viral protein synthesis reaches a maximum during the next hour and then quickly declines to almost zero by 5 h after infection. Viral RNA synthesis ceases about one hour later than protein synthesis (Baltimore et al. 1966).

Completed virions first appear in the infected cell about 3 h after infection and increase exponentially thereafter for about 2 h. Newly-synthesized RNA is quickly encapsidated into mature virions (Darnell et al. 1961), in contrast to the proteins which tend to form a precursor pool from which virions are assembled (Penman, Becker & Darnell, 1964). Assembly of the virion is not well understood but appears to proceed via the empty capsid (or top component) which is formed before association with the genome (Jacobson & Baltimore, 1968a). The empty capsids have a different protein composition to the completed virions, and it has been suggested that the entry of the RNA molecule into the structure is accompanied by a maturation cleavage of one of the proteins (VP0) to produce two of the proteins found in virions, VP2 and VP4 (Jacobson & Baltimore, 1968a). The mechanisms of such a
scheme are difficult to envisage, but evidence supporting an alternative for the assembly of virions has not yet been presented.

Other events occur late in infection such as the suppression of DNA synthesis, cytopathic affects and the rupture of the cell membrane. None of these events affect the growth of the virus except that the liberation of the progeny virions is facilitated.

(c) Integration of the synthetic processes

The various synthetic processes in the infected cell overlap considerably and the need for a regulatory mechanism has been amply demonstrated (Cooper et al. 1973). One molecule of RNA can theoretically be synthesized far quicker than a structural unit of protein, yet the final virion ratio of protein units to RNA is 60 : 1. The role of the hypothetical regulator is to reverse this disparity in synthetic ratio and to allow the accumulation of products in the correct ratio for virion assembly. The presence of the regulator also allows adequate explanation for the changes in synthetic priorities during the cycle. The infecting RNA molecule must gain some selective advantage to allow first translation and later transcription to establish the infection. Thus, the three molecules synthesized during infection (protein, complementary RNA and virion RNA) are initially made alternately, and later together in a regulated manner.

1.3 The Analysis of Polypeptides in Poliovirus-infected Cells

Early studies of the properties of poliovirus-infected cells were limited to the relatively crude analysis offered by immunological techniques. The later development of the SDS-gel electrophoresis method for the separation of
polypeptides allowed radioactive tracers to be fully exploited. Since this general technique is extensively used later in this thesis, a consideration of some aspects is included here.

(a) **Principles of SDS-gel electrophoresis**

Proteins dissolve in aqueous solutions of SDS by binding a fixed amount of SDS to the hydrophobic regions of the peptide chain (Pitt-Rivers & Impiombato, 1968). Some proteins require heating at 100°C to effect total denaturation (Maizel, White & Scharff, 1968) but once dissolved remain that way while excess SDS is in solution. The studies of Reynolds & Tanford (1970) showed that the polypeptide - SDS complex behaves as a rodlike particle, the length of which varies uniquely with the molecular weight of the polypeptide moiety. The binding of SDS in such a complex negates any intrinsic charge of the polypeptide and the migration of the whole in an electric field is determined solely by the charged SDS anions (Maizel, 1971). Thus, when SDS binds to polypeptides in a constant ratio, the charge per unit mass must be approximately constant, and such charged complexes may be separated on the basis of their molecular weight. Acrylamide gels are uniform molecular sieves and form suitable matrices in which SDS-polypeptide complexes may be electrophoresed (Shapiro, Viñuela & Maizel, 1967; Weber & Osborn, 1969; Dunker & Rueckert, 1969). Some precautions are necessary with the technique to obtain reliable results and these have been adequately reviewed by Maizel (1971).

Several recent investigators have used concentrated urea solutions in addition to SDS as a solvent for electrophoretic separations of polypeptides (Zweerink &
Joklik, 1970; Russell & Skehel, 1972; Bachrach & Hess, 1973). No theoretical explanation has been given for separations in such conditions, but it has been assumed to be similar to that for the use of SDS alone. Urea solutions tend to disrupt regions of proteins held by hydrogen bonds and so may complement the action of SDS which attacks principally hydrophobic regions. How these two protein denaturants together interact with polypeptides is not known. An attempt is made later in this thesis to explain different electrophoretic separations obtained with such a system.

(b) The detection of poliovirus polypeptides in acrylamide gels

The most commonly used method for detection of bands of protein in acrylamide gels is to use a protein-specific dye such as Coomassie Blue. This method is suitable when adequate amounts of the sample are available, and when the sample is not too complex to allow the detection of all components. The polypeptides synthesized in poliovirus-infected cells cannot be detected in this way as the presence of host proteins obscures newly-synthesized viral proteins.

The selective radioactive labelling of viral proteins has been achieved in conditions where host protein synthesis has been prevented (Summers, Maizel & Darnell, 1965). Proteins so labelled were separated on SDS-acrylamide gels and the cylindrical gels then sectioned transversely. The radioactive content of each fraction was then quantitated by scintillation counting, and a profile drawn to represent the labelled polypeptides. This method of detection has been used almost exclusively in subsequent reports on the synthesis of poliovirus polypeptides. It has the advantages
that double isotopic labelling may be used, and that $^3$H-labelled precursors are readily available and can be efficiently detected. However, the resolution of closely-migrating bands provided by this method is inferior to that obtained by the visual examination of stained gels.

Autoradiography is an alternative method for the detection of radioactively-labelled polypeptides in acrylamide gels (Fairbanks, Levinthal & Reeder, 1965). It allows resolution comparable to that of stained gels, but suffers in that low energy $\beta$-emitters such as $^3$H cannot be used. The recent perfection of methods which allow satisfactory autoradiography of dried acrylamide gel slices (Russell & Skehel, 1972) has made this method a feasible alternative to examine the polypeptides of poliovirus-infected cells labelled with $^{35}$S-methionine. It seemed likely that the improved resolution provided by this technique would allow the detection of more poliovirus polypeptides than had been found by the previous method. Preliminary experiments confirmed this suspicion, and provided the basis for much of the experimental work reported in this thesis. After this work was begun a short report appeared using autoradiography of a dried acrylamide gel slab to detect labelled polypeptides from poliovirus-infected cells (Summers et al. 1972).

1.4 The Synthesis of Poliovirus Polypeptides

Almost all new information concerning the synthesis of poliovirus polypeptides has originated since 1965 from the laboratories of Summers and Maizel, and Baltimore. The pioneering work of Maizel in developing a method by which radioactively-labelled virus polypeptides could be solubilized and analysed from a complex mixture opened a
whole new era in the study of animal viruses. Many of the basic methods and approaches used in the early studies of poliovirus polypeptides have since been successfully applied to solve problems in many areas of biology.

(a) The polypeptides of poliovirus-infected cells

Early electrophoretic separations of purified poliovirus by detergent-free methods revealed 4 polypeptide chains in the virion (Maizel, 1963). Two years later, the technique of SDS-gel electrophoresis was perfected although the basis for the separations was not understood at that time. To exploit this new method of analysis, experimental conditions were chosen in which host cell protein synthesis appeared to be minimal (Summers et al. 1965). Cells were infected at a high multiplicity in the presence of actinomycin D to prevent host RNA synthesis. Guanidine was included in the culture medium to suppress viral RNA synthesis and to allow the early replicative events including the suppression of host protein synthesis to proceed uniformly. Upon its removal, viral synthesis was expected to be synchronized and the detection of viral proteins uncomplicated by host contributions.

One hour after reversal of guanidine suppression, infected cells were labelled with a mixture of $^{14}$C-amino acids for three hours. Cytoplasmic extracts were prepared and dissolved by the addition of a solubilizing solution containing SDS as the principal agent. The labelled polypeptides were then separated by the now familiar method of electrophoresis in a cylindrical SDS-containing polyacrylamide gel. Assay of the radioactivity in transverse slices of this gel provided a profile in which 14 peaks were detected, 4 of
which corresponded to the peaks found in radioactively-labelled virions analysed similarly (Summers et al. 1965). This important experiment remains the most complete single analysis of the polypeptides of poliovirus yet published. Almost all advances that have been made subsequently in the understanding of poliovirus polypeptide synthesis have used this same overall procedure for the analysis of polypeptides.

Soon after it was realised that the molecular weights of polypeptides were related to their electrophoretic mobilities in SDS-acrylamide gels (Shapiro et al. 1967) it became obvious that the number of polypeptides made in poliovirus-infected cells greatly exceeded the coding capacity of the genome. When this observation was coupled with the finding by Summers et al. (1965) that the relative radioactive content of some polypeptides varied with the duration of labelling, the concept of proteolysis during the formation of poliovirus proteins became apparent. Three laboratories quickly developed this idea. Summers & Maizel (1968) showed that the largest polypeptide (NCVP1) was heavily labelled during a short pulse, but lost most of its radioactivity during the subsequent 45 min. Conversely, some of the structural polypeptides were not labelled during a short pulse, but accumulated radioactivity at later times. Other polypeptides did not show much change in radioactive content during a chase period, and were assumed to be stable products. Holland (1968) and Holland & Kiehn (1968) were able to extend this observation to include other picornaviruses and also to show that it was not dependent on the type of host cell. Jacobson & Baltimore (1968b) demonstrated that some of the proteolytic cleavages could be prevented by the incorporation of amino acid analogues. A new large polypeptide, NCVPO,
could be retained in detectable amounts and the cleavage of NCVP1 could be prevented. The fact that no capsid proteins appeared in these conditions led to the proposal that they were not primary gene products, but were cleaved from NCVP1. Confirmation of this observation was provided by the analysis of tryptic digests of individual polypeptides (Jacobson et al. 1970; Summers, Roumiantzeff & Maizel, 1971).

Jacobson et al. (1970) extended their approach of interfering with the cleavage mechanism to show that a general inhibitor of proteases, di-isopropyl fluorophosphonate (DFP), could cause the accumulation of abnormally large poliovirus polypeptides. This experiment provided the first evidence that proteolytic enzymes might have been responsible for the observed cleavages. This approach was recently extended by Summers et al. (1972) who showed that specific inhibitors of trypsin and chymotrypsin, L-tosyl-lysyl chloromethyl ketone (L-TLCK), L-tosyl-phenylalanyl chloromethyl ketone (L-TPCK) and L-carbobenzyloxy-phenylalanyl chloromethyl ketone (L-ZPCK), could all interfere with the cleavage mechanism. The D-isomer of the latter also was effective (although it does not inhibit chymotrypsin) and this finding suggested either that the action of the compounds may be non-specific in the infected cell, or that new proteases susceptible to D-ZPCK were at least partially responsible for the cleavage. The real meaning of these experiments is not yet known.

Korant (1972) also showed that L-TPCK and L-TLCK could interfere with cleavage, but claimed that such effects were dependent on the origin of the host cells. He failed to show any effect of L-TPCK on the cleavage process in HeLa
cell, a result in contradiction with that of Summers et al. (1972).

The use of higher concentrations of amino acid analogues allowed Jacobson et al. (1970) to detect a very large polypeptide (about 200,000 daltons) in infected cells which they claimed to be the primary translation product of poliovirus RNA. Since the size of this 'polyprotein' roughly corresponded with the coding potential of the genome, it provided more evidence that only one site existed on poliovirus RNA for the initiation of protein synthesis.

The use of p-fluorophenylalanine to restrict cleavage in infected cells led to the detection of two poliovirus polypeptides not previously recognised. Jacobson & Baltimore (1968b) found that a polypeptide that normally comigrated with VP1 was insensitive to the effect of p-fluorophenylalanine. This polypeptide (NCVPX) was shown by analysis of tryptic peptides to be distinct from VP1 (Jacobson et al. 1970). Similarly, amino acid analogues and DFP could cause a large increase in radioactivity of a peak migrating between NCVP1 and NCVP2 (Jacobson et al. 1970). This polypeptide was named NCVP1½, and improved gel techniques later showed it to be a minor component that could be found in infected cells without modification of the cultural conditions (Summers et al. 1971; Taber et al. 1971).

Another way in which the poliovirus cleavage mechanism could be impaired was to raise the temperature of the infected cells immediately before labelling (Baltimore, 1971). Polypeptides so synthesized at 43°C were only partially cleaved even during a very long chase period. Incubation of the culture at 37°C following a pulse label at
43°C also failed to permit these cleavages which suggested that some cleavage loci must be folded at 43°C in a way inaccessible to the responsible proteases (Baltimore, 1971).

Maizel, Phillips & Summers (1967) examined the polypeptide composition of the two poliovirus structural components, the virion and the empty capsids. The top component (or empty capsids) which sediments at a lower density in caesium chloride gradients than virions, contains the polypeptides VP0, VP1 and VP3, while the whole virions contain VP1, VP2, VP3 and VP4. In partially purified virion preparations, small amounts of VP0 are sometimes present (Maizel et al. 1967; Phillips & Fennell, 1973) but are removed by banding in caesium chloride gradients. The possible role of the top component in the morphogenesis of poliovirus was studied by Jacobson & Baltimore (1968a). Using guanidine to inhibit temporarily the movement of progeny RNA into completed virions they were able to show that material accumulated in the region of the top component. On release from guanidine inhibition, virions accumulated and the top component pool diminished. On the basis of these experiments, Jacobson and Baltimore proposed that the final encapsidation of progeny RNA is accompanied by cleavage of the polypeptide VP0 to give VP2 and VP4. Because the polypeptide VP0 appeared to have a central role in structural units, Jacobson and Baltimore so named it instead of its former name NCVP6 (Summers et al. 1965). The terminology, VP0, will be used throughout this thesis.

Several reports have claimed that more than 4 polypeptides comprise the poliovirion (Vanden Berghe & Boeyé, 1972; Phillips & Fennell, 1973; Wright, 1973). Also, Cooper,
Summers & Maizel (1970) have reported two components representing VP2 in analyses of cytoplasmic extracts of infected cells. All of these reports were based on the detection of multiple bands in SDS-acrylamide gels and all concerned the region in gels around the polypeptides VP2 and VP3. Also, all samples used for analysis in these reports were treated at some time with urea solutions, either dilute (0.5 M) or concentrated (5 M). Work to be described later in this thesis suggests that such multiple banding may be caused by some urea solutions, and that it is likely that only four polypeptides exist in the poliovirion.

(b) The in vitro synthesis of poliovirus polypeptides

Poliovirus polypeptides can be synthesized in a cell-free system derived from membrane-bound poliovirus-specific polysomes (Roumiantzeff, Summers & Maizel, 1971). The product analysed in SDS-acrylamide gels comprised a broad peak of radioactivity in the high molecular weight region (about 200,000 daltons) and a heterogeneous collection of lower molecular weight polypeptides. No prominent peaks corresponded with poliovirus polypeptides synthesized in vivo. These results suggested that if translation was being faithfully completed, the products were not being specifically cleaved in the cell-free system. However, faithful translation (as revealed by tryptic peptide analysis) of another picornaviral RNA has been achieved in an animal cell-free system supplemented with encephalomyocarditis RNA (Kerr, Brown & Tovell, 1972). This system has been used to provide evidence showing that the mechanism of action of interferon is at the level of translation of viral RNA to protein (Friedman et al. 1972).

Rekosh, Lodish & Baltimore (1970) endeavoured
to get direct translation of the poliovirus genome in a cell-free bacterial system derived from *Escherichia coli*. In contrast to the previous approaches, no high molecular weight polypeptides were detected using this system. All products were smaller than about 60,000 daltons which suggested either that translation products were being quickly broken down, or that the system was able to recognise more than one codon for the initiation of protein synthesis. In the case of the latter, it was not known if the message was being translated in phase, or whether each initiating codon was followed later by a complementary terminating codon. The usefulness of all of these *in vitro* systems for the translation of poliovirus polypeptides appeared to be limited.

(c) **The molecular weights of the poliovirus polypeptides**

Estimation of relative mobilities in SDS-acrylamide gels is the only feasible method at present available to measure the molecular weights of the polypeptides of poliovirus. Since this method was first described (Shapiro *et al.* 1967), it has been closely studied in order to check its reliability (Weber & Osborn, 1969; Dunker & Rueckert, 1969). Irregularities have been observed in both high and low molecular weight ranges, and also with particular proteins (Dunker & Rueckert, 1969). Nevertheless, the method has many advantages and in many cases, is the only one available. Dunker & Rueckert (1969) and Maizel (1971) have suggested a limit of accuracy of about 10% for the determination of molecular weights by this method.

Two reports have appeared assigning molecular
weight values to many of the poliovirus polypeptides (Maizel & Summers, 1968; Jacobson et al., 1970). These values are in agreement for the sizes of the capsid polypeptides, but differ for higher molecular weight polypeptides (about 100,000 daltons) where the values of Jacobson et al. (1970) are considerably larger. Such variations could have occurred for several reasons. Firstly, the choice of adequately-characterized proteins to use as standards is essential for such determinations. Secondly, it has been pointed out by Fish, Reynolds & Tanford (1970) and acknowledged by Maizel (1971) that all inter- and intra-chain disulphide bonds need to be reduced in the presence of SDS in order to allow polypeptides to migrate as a function of their size. Thirdly, the migration distances of standards and unknowns should be measured under the same load conditions on the same gel after identical sample preparation to minimize irregularities. With both of the previous determinations of the molecular weights of poliovirus polypeptides, very little data is presented to show that these three points were rigidly observed. Thus, doubt exists that either of the previously-determined sets of values are as close to the true values as the method will allow. A knowledge of accurately-determined molecular weights of poliovirus polypeptides is essential to correlate the translation products with the genetic coding potential, and also to establish the cleavage pathway. Thus, an attempt is made later in this thesis to overcome some criticisms of previous determinations, and to obtain molecular weight values believed to be closer to the real ones.

1.5 Aspects of Polypeptide Synthesis and Processing in Eucaryotes
Most of the information concerning the mechanisms of protein synthesis has been established with bacterial systems. However, many similarities exist in eucaryotic systems. Recent reviews on the synthesis of proteins adequately cover the subject (Lengyel & Söll, 1969; Lucas-Lenard & Lipmann, 1971; Haselkorn & Rothman-Denes, 1973). Aspects most pertinent to this thesis are the initiation of messenger translation, and the post-translational cleavage of polypeptide chains. Both of these topics are therefore emphasised in the discussion below.

(a) **Initiation of protein synthesis in eucaryotic systems**

Initiation of protein synthesis in eucaryotes involves the interaction of the small ribosomal subunit with an AUG triplet on a suitable messenger RNA and the simultaneous binding of a molecule of methionyl-tRNA\(^\ast\) to form a complex (Smith & Marcker, 1970; Brown & Smith, 1970). This complex formation requires the activities of three initiation factors as well as an energy source supplied by GTP (Shafritz & Anderson, 1970; Pritchard \textit{et al.} 1970). Subsequent steps involve the binding of the larger ribosomal subunit and the transfer of the methionyl-tRNA\(^\ast\) to the peptidyl site on it. This step concludes the initiation process and the following process of elongation begins. Very simply, other charged transfer RNA molecules bind in succession to the amino acyl site on the ribosome, are transferred to the peptidyl site during the formation of the peptide bond and displace the preceding discharged transfer RNA. This process thus places methionine at the N-terminus of all growing peptide chains. It may not remain there, however, as an amino-peptidase
frequently removes it and sometimes the penultimate amino acid also to expose the N-terminus actually found.

The procaryotic initiation process differs from that described above principally in that the initiating methionyl-tRNA\textsubscript{f} is formylated enzymatically prior to the formation of the first complex. This formyl methionyl-tRNA\textsubscript{f} positions formylated methionine exclusively at the N-terminus of newly-synthesized polypeptides. Subsequently, a formyl transferase enzyme removes the formyl group from the growing peptide, exposing methionine at the N-terminus which may or may not be removed in a fashion similar to that found in eucaryotes.

One unexpected similarity between the two systems was that the methionyl-tRNA\textsuperscript{\textregistered} from eucaryotes could be artificially formylated by a procaryotic enzyme (Caskey & Redfield, 1967). Even more surprising was the observation of Housman et al. (1970) that such a formylated tRNA could initiate protein synthesis in eucaryotic cell-free systems, and position N-formylmethionine at the N-terminus of newly-synthesized chains. In such cases, the formyl group could not be removed as eucaryotic cytoplasm does not contain a suitable formyl transferase enzyme. It followed that aminopeptidases were therefore unable to remove the blocked methionine residue and so a method for labelling the initiation site of newly-synthesized polypeptides was available. Part of the experimental work of this thesis endeavoured to utilize this finding in order to determine in vivo the N-terminal portion of the poliovirus translation product(s).
(b) Post-translational cleavage of polypeptides

All aspects of the structure of a protein contribute to its function, some of which may be the result of modifications after translation. The only such modification known to occur during picornavirus synthesis is one involving extensive proteolytic cleavage of the translation product (Summers & Maizel, 1968; Jacobson & Baltimore, 1968b). The mechanism and extent of this process in uninfected mammalian cells is therefore considered.

One function of post-translational cleavage in mammals is to delay the activation of functional proteins. Examples of this process are in the activation of zymogens to produce proteolytic enzymes, the activation of two agents, prothrombin and fibrinogen, involved in blood clotting, and the conversion of proinsulin to insulin. Most evidence suggests that these molecules are cleaved extracellularly by ubiquitous proteases, although proinsulin may be cleaved intracellularly (Steiner et al. 1969). Less well characterized examples of proteolysis occur intracellularly during times of nutritional starvation, tissue involution and cell protein turnover. These latter examples demonstrate the principle of limited proteolysis where only some of the seemingly susceptible peptide bonds in native substrates are attacked. The combined data of many experiments reviewed by Schimke (1970) suggests that the determining factor for such proteolysis is the steric conformation of the substrate, rather than any chemical factors or specificity of the proteases. This scheme would permit regulation of proteolysis by allowing the alteration of conformations with small molecules, or the evolution of stable protein configurations.
for those proteins requiring little degradation. Such a substrate-determining system would have selective advantages as only a small number of proteases would be necessary for the complete degradation of unwanted proteins.

Proteolytic enzymes are widespread in animal tissues, particularly in lysosomes. The cathepsins are a group of such proteases which have complementary specificities and so are able to effect a total digestion of proteins (Barrett, 1969). Such enzymes are normally restricted to the lysosomes but are readily released in the case of cell death. Other proteases must be present outside the lysosomes to explain the dynamic protein turnover continually occurring in mammalian cells (Pine, 1972).

Consideration of the proteolysis during the synthesis of picornavirus polypeptides now suggests that the requisite enzymes for cleavage are probably present in all host cells and that the specificity of cleavage is governed by the physical conformation of the translation product(s) rather than the activity of a specific enzyme. Support for these conclusions comes firstly from the observations that cleavage is consistent in cells derived from different species which presumably contain different batteries of proteases (Holland, 1968; Kiehn & Holland, 1970; Laskey, Gurdon & Crawford, 1972), and secondly from experiments showing that changes in the physical state of the translation products caused by mutation (Cooper, Summers & Maizel, 1970) or increased temperatures (Baltimore, 1971) lead to altered cleavage specificities. Also, the fact that the same specific inhibitors of proteolytic enzymes can restrict the cleavage of picornavirus polypeptides (Summers et al., 1971) and also
cause the accumulation of some high molecular weight precursors in uninfected cells (Taber, Wertheimer & Goldrick, 1973) further suggests that picornaviruses use proteases already present in their host cells rather than synthesize their own. The possibility of a virus-coded protease to effect cleavage seems unlikely as no room is left in the poliovirus genome after accounting for the other known functions (Cooper et al. 1971).
CHAPTER TWO

METHODS AND MATERIALS

2.1 Cell Cultures
   (a) Propagation of cells
   (b) Infection of cells
   (c) Growth and labelling of cells
   (d) Measurement of incorporated radioactivity by cultures

2.2 Poliovirus Stocks

2.3 Preparation of Radioactive Virus and Empty Capsids

2.4 Polyacrylamide Gel Electrophoretic Analysis
   (a) General procedure
   (b) Sample preparation
   (c) Autoradiography
   (d) Staining
   (e) Detection of radioactivity by scintillation counting
   (f) Determination of molecular weights of polypeptides

2.5 Preparation of Cytoplasmic Extracts by Dounce Homogenization

2.6 Analysis of Poliovirus RNA in SDS-Sucrose Gradients

2.7 Preparation and Analysis of Tryptic Digests of Individual Polypeptides

2.8 Analysis of Amino Acids by Paper Chromatography

2.9 Preparation of N-formyl-\textsuperscript{35}S-methionine
2.10 Preparation of Transfer RNA from Cells
2.11 Analysis of Ribonuclease Digests of Transfer RNA by Paper Electrophoresis
2.12 Sources of Reagents
2.1 Cell Cultures

(a) Propagation of cells

Cells used for the growth of poliovirus 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 & Jimbo, 1968), supplemented with 10% calf serum, 1.25 mg/ml NaHCO$_3$, 146 µg/ml L-glutamine and 100 µg/ml each of penicillin, streptomycin and neomycin. Cells were grown at 37°C in monolayers on the inner surface of rotating (5 rev/h) 2.25 litre round bottles, seeded with 16 x 10$^6$ cells and harvested 5 days later when the cell number was approximately 120 x 10$^6$. Cells were removed from the glass by a 5 min incubation at 37°C with 30 ml of a solution containing 0.025% trypsin, 0.02% disodium ethylenediaminetetra-acetate (EDTA), 0.2 mg/ml NaHCO$_3$, 8 mg/ml NaCl, 0.4 mg/ml KCl, 60 µg/ml Na$_2$HPO$_4$ and KH$_2$PO$_4$. 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 growth of the cells were done by technical staff of the department.

(b) Infection of cells

Cells not used immediately after harvesting were suspended in Eagle's medium overnight (6 x 10$^5$ cells/ml) by gentle stirring at 37°C. Such cells were harvested by centrifugation and resuspended in poliovirus seed (see Section 2.2) at a concentration greater than 3 x 10$^7$ cells/ml, and maintained with gentle stirring for 2 h at 0°C. The multiplicity of infection ranged from 30 to 50 plaque forming units (p.f.u.)
per cell.

(c) Growth and labelling of cells

Infected cells (after the removal of residual seed virus) or uninfected cells were resuspended in Eagle's medium supplemented with 2% calf serum and 0.9 mg/ml NaHCO$_3$, but without added methionine, at 3 x 10$^6$ cells/ml. Particular cultures may have lacked all added amino acids or phosphates as indicated in the text. Cultures were gently stirred at 37°C until additions of $^{35}$S-methionine (or other isotopic precursors) were made. The specific activity of the $^{35}$S-methionine varied from 20 to 55 Ci/mMole, and the radioactive concentration used in cultures varied between 5 and 12 μCi/ml for all experiments reported except those in Chapter 7. In general, cultures labelled for longer times had lower radioactive concentrations added to them, while those labelled for short pulses contained 10 or 12 μCi/ml. Cultures that were pulse-labelled were chased with an equal volume of Eagle's complete medium containing 2 mg/ml unlabelled methionine.

(d) Measurement of incorporated radioactivity by cultures

The incorporation of radioactive precursors into acid insoluble material was generally measured by sampling 10 or 25 μl volumes of culture on to a 2 cm Whatman's filter paper disc and immediately immersing the disc in 5% (w/v) trichloracetic acid at 0°C. At least 10 ml of acid was used for each sample to be precipitated. Discs were gently agitated in 3 changes of ice-cold acid, 2 changes of ice-cold ethanol and 2 changes of ice-cold ether. They were then dried, immersed in 5 ml of toluene-based scintillation fluid
containing 0.5% PPO and 0.05% POPOP and the radioactive content determined (with the optimum channel and gain settings for the isotope concerned) in a Packard Tri-Carb Liquid Scintillation Spectrometer.

2.2 Poliovirus Stocks

All virus used was poliovirus type I, strain Mahoney. To prepare virus stocks, a cell suspension was infected with virus at a multiplicity of 30 p.f.u./cell and incubated at 37°C in growth medium at 2 x 10^6 cells/ml for 6 h. The whole culture was adjusted to pH 5 with HCl, the cells gently suspended by centrifugation and two-thirds of the supernatant medium removed and discarded. The cells were then frozen at -20°C, thawed, and the cell debris removed by centrifugation at 10,000 g for 10 min. The supernatant was dispensed in 5 ml portions and stored frozen at -20°C. Two such stocks were used in this report and contained 2 to 3 x 10^9 p.f.u./ml. Virus stocks were assayed by the agar cell-suspension plaque technique of Cooper (1961) and were done by Miss J. Mundy.

2.3 Preparation of Radioactive Virus and Empty Capsids

Cells were infected and incubated in medium without added methionine. At 3 h after infection, ^35S-methionine was added to 5 μCi/ml and incubation continued for 3 h. The culture was adjusted to pH 5, frozen at -20°C, thawed, and the cell debris removed by centrifugation at 10,000 g for 10 min. The supernatant was centrifuged for 1 h at 200,000 g and the pellets gently dispersed at room temperature in a total of 1 ml 0.1% SDS in RSB (0.01 M-NaCl, 0.0015 M-MgCl₂, 0.01 M-Tris pH 7.4). Virus suspensions were layered on to 5 - 20% (w/w) linear sucrose gradients and
Fig. 2-1 Sedimentation through a 5-20% sucrose gradient of a preparation of $^{35}$S-methionine-labelled virus. Such preparative gradients were centrifuged for 4 h at 55,000 g. Points represent the total radioactivity in 10 µl of each fraction.
centrifuged for 4 h at 55,000 g. The gradients were fractionated into 0.5 ml fractions and the total radioactivity in 10 µl of each fraction determined. A typical gradient is shown in Fig. 2-1 where the major peak of virus and the minor peak of naturally-occurring empty capsids can be seen. Samples for electrophoresis were taken from the peak fractions.

2.4 Polyacrylamide Gel Electrophoretic Analysis

(a) General procedure

The electrophoretic procedure used was essentially the continuous SDS-phosphate method of Maizel (1971) with the modifications used by Russell & Skehel (1972). Both acrylamide monomers were recrystallized before use according to Loening (1967). Gels contained 6 - 12% acrylamide with a constant ratio of N, N'-methylenebisacrylamide at 2.7% of the acrylamide concentration, 0.1% SDS, 0.05 M-sodium phosphate buffer pH 7.2, 5 M-urea and 0.01 M-EDTA. Gels were polymerized at room temperature with 0.1% TEMED and 0.05% ammonium persulphate in perspex tubes 15 cm long with an internal diameter of 6.5 mm. The concentration of urea in the gels was varied in some experiments as indicated in figure legends. The additions of other reagents in experiments in Chapter 5 are indicated in the text.

Electrophoresis buffer contained 0.05 M-sodium phosphate buffer, 0.01 M-EDTA and 0.1% SDS. Electrophoresis was generally for 16 h at 50 volts drawing a current of 4 mA/gel. Gels with higher concentrations of acrylamide, or in which high molecular weight polypeptides were being examined were electrophoresed at potentials up to 70 volts.
(b) **Sample preparation**

Samples were generally prepared from whole cells by dissolving $6 \times 10^6$ cells in 0.4 ml of solution containing 2% (w/v) SDS, 5% (v/v) 2-mercaptoethanol and 7.5 M-urea and heating for 2 min in a boiling waterbath. Occasions on which urea was excluded from the disrupting fluid are indicated in the text. Samples of virus (or empty capsids) from sucrose gradients and cytoplasmic extracts were added to an equal volume of the disrupting fluid and heated as previously described, prior to electrophoresis. Samples to be detected later by autoradiography contained 50 - 200 x $10^3$ counts per minute $^{35}S$ contained in volumes usually less than 50 µl.

(c) **Autoradiography**

After electrophoresis, gels were sliced longitudinally using a device similar to that described by Fairbanks et al. (1965). Slices were washed in 20 ml volumes of 7% (v/v) acetic acid for 1 h, laid on a porous sheet of linear polyethylene, 45 µm porosity (Bel-Art Products, New Jersey) covered with wet cellophane and dried under reduced pressure overnight at room temperature. The dried strips of gel which adhered to the cellophane were then exposed for 2 - 7 days to medical X-ray film (Kodak RP Royal X-Omat) and developed in regular film processing chemicals (Kodak Pty. Ltd., Melbourne).

(d) **Staining**

Gels were sliced longitudinally (as for autoradiography) when polypeptides were to be detected by staining. Slices were immersed in 0.5% Coomassie Blue in 7% (v/v) acetic acid for 1 h at 37°C and destained in changes
of 7% acetic acid for 2 days at 37°C. Gels could then be dried and autoradiographed as previously.

(e) Detection of radioactivity by scintillation counting

This method was used in one experiment described in Chapter 7. Electrophoresis conditions were those used by Summers et al. (1965) and differ principally from those described above in that urea was used in the gels at 0.5 M. The gel was removed from the tube, frozen and fractionated transversely into 1.6 mm slices. Each slice was placed in a vial, 0.5 ml 10% (v/v) piperidine added, the slices dried at 60°C, 0.5 ml water added and followed 2 h later by 10 ml dioxane-based scintillation fluid (15% w/v napthalene, 0.23% PPO and 0.009% POPOP in dioxane). Samples were counted in a Packard Scintillation Spectrometer with settings to differentially detect \( ^{35}S \) and \(^3H\) emissions. After correction for spillover of emissions from \( ^{35}S \) into the \(^3H\) channel, the data were plotted as shown in Fig. 7-4.

(f) Determination of molecular weights of polypeptides

Except where otherwise stated (Chapter 5, Section 5.2), molecular weights of polypeptides were determined in SDS-acrylamide gels that also contained 5 M-urea. Molecular weights assigned to poliovirus polypeptides were derived from a plot of the logarithm of the molecular weight against the distance of electrophoretic migration (Shapiro et al. 1967) relative to standard proteins of known molecular weight. The molecular weights of the marker proteins used were as follows: rabbit skeletal muscle myosin prepared as described by Perry (1955); 212,000 daltons (Gershman, Stracher &
Driezen, 1969); haemocyanin of Jasus lalandii, 86,000 daltons (Parish & Marchalonis, 1970); human serum albumin, 66,000 daltons (Low, 1952); pepsinogen, 39,000 daltons and pepsin, 33,600 daltons (Fruton, 1971); trypsin, 23,800 daltons (Keil, 1971); tobacco mosaic virus protein, 17,650 daltons (from the amino acid sequence in "Atlas of protein sequence and structure", vol. 5).

The three largest proteins were iodinated with $^{125}$I using the method of Greenwood, Hunter & Glover (1963). After completion of the reaction, the proteins were made 1% with respect to SDS and excess iodide was removed by dialysis against 0.1% SDS. Proteins were then prepared as previously for electrophoresis, about 50,000 counts per minute $^{125}$I requiring an exposure time for autoradiography of 2 days.

2.5 Preparation of Cytoplasmic Extracts by Dounce Homogenization

The procedure was done at ice temperature. Cells were washed twice with ice-cold PBS and then RSB was added to give a cell concentration of 2.5 x 10^7/ml. After 10 min equilibration, the cells were broken by 15 strokes in a glass Dounce homogenizer and the nuclei removed by centrifugation (800 g for 10 min). The supernatant cytoplasmic extract was then treated as required.

2.6 Analysis of Poliovirus RNA in SDS-Sucrose Gradients

Cytoplasmic extracts of poliovirus-infected cells were either made 1% with respect to SDS and heated for 2 min at 60°C, or were made up to 0.3 M-NaCl and 0.03 M-sodium citrate (pH 7.5) and pancreatic ribonuclease (10 μg/ml) added for 10 min at room temperature prior to SDS treatment. Extracts were layered on to linear 15 - 50% (w/w) sucrose
gradients in 0.1 M-NaCl, 0.01 M-tris HCl pH 7.4, 0.01 M-EDTA and 0.5% SDS. Following centrifugation for 16 h at 55,000 g in a Spinco SW 25.3 rotor at 20° C, the gradients were fractionated through a Gilford continuous recording spectrophotometer into 0.6 ml fractions. The spectrophotometer monitored the optical density at 260 nm, indicating the sedimentation positions of 28 S and 16 S ribosomal RNA. 50 μl volumes of each fraction were assayed for total or acid insoluble radioactivity.

2.7 Preparation and Analysis of Tryptic Digests of Individual Polypeptides

Large quantities of some 35S-methionine-labelled poliovirus polypeptides were prepared by scaling up the analytical electrophoresis procedure described in Section 2.4. Samples for electrophoresis were either 35S-methionine-labelled virus from sucrose gradients, or SDS digests of whole cells labelled under the desired conditions. Twelve or 24 gels of each preparation were run and autoradiographs prepared as for the analytical procedure, except that gel slices were soaked in distilled water rather than 7% acetic acid before drying. The autoradiographs allowed the required polypeptides to be located exactly, and cut out from the gel slices. These gel bits were rehydrated in 0.1% SDS, frozen and thawed, broken further by forcing through the nozzle of a syringe and boiled for 1 min at 100°C. Polypeptides were eluted for 4 h at 42°C and gel bits removed by filtration through a Whatman's membrane filter of porosity 22 nm. Approximately 10^6 counts per minute 35S were recovered for each polypeptide. After addition of 1 - 2 mg lysozyme as carrier, the polypeptides were precipitated by the addition
of 2 volumes of ethanol and 20 μg NaCl. Precipitates were allowed to settle overnight at -20°C, were then resuspended in 1 ml water and reprecipitated twice more to remove SDS. Proteins were then digested at 37°C with 50 μg trypsin (TPCK treated, Worthington) for 2 h at pH 9. Samples were further digested for 4 h after the addition of another 50 μg trypsin. Digests were dried and redissolved in 50 – 200 μl volumes of pH 6.5 buffer (pyridine 10 ml, acetic acid 0.4 ml, water 120 ml). Insoluble residue was checked for solubility at pH 9. In every case, less than 5% of the radioactivity insoluble at pH 6.5 was soluble at pH 9 which suggested that all tryptic peptides were soluble in the buffer used.

Separation of the peptides by high voltage paper electrophoresis and chromatography was done as described by Laver (1969). Dried sheets were then autoradiographed to detect the methionine-containing peptides. Maps were usually developed 14 days after application of approximately 5 – 10,000 counts per minute ^35^S per expected methionine-containing peptide.

Alternatively, peptides were separated by one dimensional electrophoresis on cellulose acetate strips (Oxoid) at pH 6.5 using the buffer above, or at pH 3.5 (pyridine 10 ml, acetic acid 1 ml, water 89 ml). The apparatus and method used was described by Fazekas de St. Groth, Webster and Datyner (1963). Radioactive peptides were detected by autoradiography as above except that one-tenth of the radioactivity was required.

2.8 Analysis of Amino Acids by Paper Chromatography

Ascending chromatography was done on 20 cm x 5 cm strips of Whatman's No. 1 filter paper. Volumes up to 2 μl
were applied as a band 1.5 cm from the bottom of the strip, which was immersed 0.5 cm in the developing solvent (n-butanol 70 ml, water 28 ml, 1 M-2-mercaptoethanol 2 ml). Chromatography was continued for 3 h after which the solvent front had moved about 15 cm. The paper was dried and either fractionated into 1 cm strips and assayed directly for radioactivity by scintillation counting, or stained with ninhydrin (1% in acetone).

2.9 Preparation of N-formyl-\(^{35}\)S-methionine

\(^{35}\)S-methionine was prepared from Na\(^{23}\)S\(^{35}\)SO\(_4\) (carrier-free) by the method of Sanger, Bretscher & Hocquard (1964) except that pronase (Calbiochem) was used to digest the radioactive yeast protein. This product contained greater than 95% of its radioactivity as \(^{35}\)S-methionine (as judged by paper chromatography) and had an approximate specific activity of 30 Ci/m Mole. Chemical synthesis of N-formyl-\(^{35}\)S-methionine was based on the method of Sheehan & Yang (1957). Approximately 0.03 \(\mu\) Mole \(^{35}\)S-methionine was dried in a closed vessel in a stream of oxygen-free nitrogen. Acetic anhydride (0.35 ml) and 1 ml formic acid (both redistilled and stored under nitrogen) were added separately.

After reaction for 1 h at 5°C, the mixture was warmed to room temperature and the solvents dried off in a stream of nitrogen. The product was extracted with 0.5 ml methanol and analysed for radioactive content by paper chromatography. The formylation was always greater than 96% complete.

To ensure that the product was free of \(^{35}\)S-methionine, it was dissolved in 2% (v/v) acetic acid and passed through a column (2 cm x 0.4 cm) of Dowex 50W (Fluka,
Switzerland) ion exchange resin equilibrated with the same solvent. The eluted product contained approximately 98% of its radioactivity as either N-formylmethionine or its sulphoxide derivative. A similar column was found to effectively bind 95 - 99% of free \(^{35}\)S-methionine passed through it.

The N-formyl-\(^{35}\)S-methionine was redissolved in 3 mM 2-mercaptoethanol and used directly.

2.10 Preparation of Transfer RNA from Cells

Cytoplasmic extracts of cells were prepared as described in Section 2.5. Transfer RNA fractions were then prepared as described by von Ehrenstein (1967) and finally precipitated with ethanol.

2.11 Analysis of Ribonuclease Digests of Transfer RNA by Paper Electrophoresis

Labelled transfer RNA was digested with ribonuclease (10 \(\mu\)g/ml) and 10 \(\mu\)l of the digest applied as a band to Whatman's No. 3 MM filter paper, pre-soaked with buffer pH 3.5 (pyridine 10 ml, acetic acid 1 ml, water 89 ml). Electrophoresis was for 1 h at 2,000 volts and 150 mA. The sheet was later dried and fractionated transversely into 1 cm strips which were assayed for radioactivity by scintillation counting.

2.12 Sources of Reagents

Common chemicals not listed below were analytical grade reagents supplied by major international companies.

Acrylamide and \(N,N'\)-methylene bisacrylamide
Eastman Organic Chemicals, New York

Auto-Pow dehydrated medium
Flow Laboratories Inc., Scotland
Amino acid analogues:

- L-azetidine-2-carboxylic acid
- L-canavanine sulphate
- 3,4-dehydro-DL-proline
- DL-3,4-dihydroxyphenylalanine
- DL-norvaline
- β-2-thienylserine
- 4-aza leucine
- L-3-aminoxytosine
- Allyl-DL-glycine
- β-2-thienylalanine
- DL-β-phenyl lactic acid
- DL-4 and 6-fluorotryptophan
- DL-ortho fluorophenylalanine
- DL-meta fluorophenylalanine
- DL-7-aza tryptophan
- p-amino-DL-phenylalanine
- DL-β-phenylserine
- DL-1,2,4-triazole-3-alanine
- DL-para fluorophenylalanine
- Diphenyl carbamyl chloride
- Dowex 50W X2 (200-400 mesh)
- Emetine
- Formamide
- Hemocyanin (Jasus lalandii)

Suppliers:
- Calbiochem, California
- British Drug Houses, England
- Nutritional Biochemicals Corp., Ohio
- Sigma Chemical Co., Missouri
- Eastman Organic Chemicals, New York
- Fluka, Switzerland
- British Drug Houses, England
- Fluka, Switzerland
- Provided by Prof. G. L. Ada, Department of Microbiology, JCSMR
<table>
<thead>
<tr>
<th>Substance</th>
<th>Supplier/Description</th>
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</thead>
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<tr>
<td>Human serum albumin</td>
<td>Nutritional Biochemicals Corp., Ohio</td>
</tr>
<tr>
<td>Iodine - 125, Code IMS 3</td>
<td>The Radiochemical Centre, Amersham</td>
</tr>
<tr>
<td>L-methionine-methyl-(^{3}H) (7.5 Ci/mMole)</td>
<td>The Radiochemical Centre, Amersham</td>
</tr>
<tr>
<td>L-methionine-(^{35}S) (20-55 Ci/mMole)</td>
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</tr>
<tr>
<td>Myosin (rabbit skeletal muscle)</td>
<td>Prepared according to Perry (1955)</td>
</tr>
<tr>
<td>Pactamycin</td>
<td>A gift from Upjohn Co., Michigan</td>
</tr>
<tr>
<td>Pepsin and pepsinogen</td>
<td>Worthington Biochemical Corp., New Jersey</td>
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<tr>
<td>Phenyl methane sulphonyl fluoride</td>
<td>Sigma Chemical Co., Missouri</td>
</tr>
<tr>
<td>Phosphorus - 32 &quot;carrier-free&quot;</td>
<td>Australian Atomic Energy Commission</td>
</tr>
<tr>
<td>POPOP (1,4-bis-(5-phenyloxazole)- benzene</td>
<td>Ajax Chemicals, Sydney</td>
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<tr>
<td>PPO (2,5-diphenyloxazole)</td>
<td>J.T. Baker Co., New Jersey</td>
</tr>
<tr>
<td>Pyridine</td>
<td>Worthington Biochemical Corp., New Jersey</td>
</tr>
<tr>
<td>Ribonuclease (bovine pancreas)</td>
<td>Koch-Light Laboratories Ltd., England</td>
</tr>
<tr>
<td>Sodium dodecyl sulphate (SDS)</td>
<td>Matheson, Coleman and Bell, Ohio</td>
</tr>
<tr>
<td>Sodium sulphate-(^{35}S) (carrier free)</td>
<td>Commissariat à l'energie, France</td>
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TEMED (N,N,N',N''-tetramethyl-ethylenediamine)
TLCK (tosyl-L-lysyl chloromethane)
Tobacco mosaic virus protein
Toluene sulphonyl fluoride
TPCK (tosyl-L-phenylalanyl chloromethane)
Trypsin (TPCK treated)
Urea (analytical reagent)

Eastman Organic Chemicals, New York
Calbiochem, California
A gift from Dr. E. Benjamin, Dept. of Medical Microbiology, University of California, Davis
Aldrich Chemical Co., Wisconsin
Calbiochem, California
Worthington Biochemical Corp., New Jersey
Mallinckrodt Chemical Works, Missouri
CHAPTER THREE

THE POLYPEPTIDES OF POLIOVIRUS-INFECTED CELLS

3.1 Introduction

3.2 The Polypeptides of Poliovirus Detected by Autoradiography

(a) The transition from host to virus-directed protein synthesis
(b) The number of poliovirus polypeptides detected in infected cells
(c) A proposed system of nomenclature for poliovirus polypeptides

3.3 Proteolytic Cleavage Demonstrated by Pulse-Chase Experiments

(a) The fate of polypeptides labelled during a short pulse
(b) Quantitation of the changes in radioactive content during a chase period
(c) The absence of detectable proteolysis in uninfected cells
(d) The cleavage process at different times during the infection cycle

3.4 Apparent Differences between the Polypeptide Compositions of Whole Infected Cells and Cytoplasmic Extracts of Infected Cells

3.5 Discussion

3.6 Summary
3.1 Introduction

Poliovirus has attracted considerable recent interest in regard to the specific activity of proteases apparently involved in the production of its functional proteins. Since poliovirus is believed to inhibit completely the macromolecular synthesizing machinery of its host cell, the detection of virus-specific polypeptides has been relatively simple using radioactively-labelled amino acids. The techniques used to detect the products have, with one recent exception (Summers et al. 1972), involved the transverse fractionation of cylindrical polyacrylamide gels and the quantitation of the radioactivity in each fraction by scintillation counting. The profiles obtained by this method have been adequate to demonstrate prominently labelled polypeptides and those well separated on the gels from other species. In practice, visual observation of bands in gels (eg. staining or autoradiography) gives a more satisfactory and convincing resolution of polypeptide species than the quantitation of transverse fractions. Poliovirus polypeptides cannot be detected in infected cells by staining as they are obscured by the dense staining of the host proteins. The recent perfection of techniques that allow the autoradiography of dried acrylamide gel slices thus provided an alternative tool by which a more detailed examination of the polypeptides of poliovirus may be achieved.

This chapter demonstrates the application of this technique to examine polypeptides labelled with $^{35}$S-methionine of high specific activity. Several aspects of the interaction of poliovirus with its host cell have been extended and the results obtained form the basis for further experiments in
later parts of this thesis. After the commencement of this work, a brief report covering some aspects discussed in Chapter 4, and based on a similar autoradiographic approach has appeared (Summers et al. 1972).

3.2 The Polypeptides of Poliovirus Detected by Autoradiography

(a) The transition from host to poliovirus-directed protein synthesis

Previous investigations of the action of poliovirus on the host cell showed that, using high multiplicities of infection, detectable host protein synthesis is stopped about 3 to 4 h after infection (Summers et al. 1965; Jacobson & Baltimore, 1968a). These workers invariably used either actinomycin D to prevent host messenger RNA synthesis, or inhibitory concentrations of guanidine for periods up to two hours after infection to suppress viral RNA synthesis and so synchronize the shut down of host protein synthesis. Neither of these two inhibitors were used in any experiments in this thesis. The first experiment described shows that conditions of infection can be chosen without the use of drugs such that host protein synthesis is prevented and the polypeptides being synthesized appear to be virus-specific.

Aliquots of an infected cell culture were labelled with $^{35}$S-methionine for 20 min intervals at several times from 1 to 3 h after infection. Samples from each aliquot together with an uninfected cell sample were analysed by acrylamide gel electrophoresis and an autoradiogram prepared (Fig. 3-1). Host polypeptides continue to be detected until 2.5 h after infection, but not thereafter. Meanwhile, new polypeptides assumed to be virus-specific are
Fig. 3-1 An autoradiogram of acrylamide gel slices showing the transition from host-directed to poliovirus-directed protein synthesis. Cultures described in the text were labelled for 20 min with 5 µCi/ml $^{35}$S-methionine (30 Ci/mMole). (In all subsequent experiments described in this thesis, similar concentrations of $^{35}$S-methionine were used to label cultures prior to preparation of samples for electrophoresis and autoradiography). Equal volumes of extracts equivalent to $3 \times 10^5$ cells were analysed in 12% gels. The numbers indicate the time (in hours) after infection at which labelling was done.
first detected at 2 h after infection and increase thereafter, reaching a peak later than 3 h after infection. It was concluded that samples analysed later than 3 h after infection should reveal only virus-specific proteins. Similar experiments showed this pattern of protein synthesis to be reproducible, and that the time at which maximum viral protein synthesis was proceeding was 3.5 to 4 h after infection. In many subsequent experiments this period was chosen for the labelling of virus-specific proteins. The identity of the viral polypeptides revealed in Fig. 3-1 will be examined in subsequent sections below.

Inspection of Fig. 3-1 reveals that as infection proceeds all host polypeptides apparently decline at an equal rate. This aspect could not be accurately quantitated due to the lack of resolution of the large number of host polypeptides present. However, if visual quantitations of photographic density are an adequate guide, it seems that the mechanism of inhibition of host protein synthesis acts equally on all species. This observation is consistent with two current proposed mechanisms for the shut down of host protein synthesis in picornavirus-infected cells; the effect of double-stranded RNA on initiation of translation (Ehrenfeld & Hunt, 1971) and the regulatory action of the proposed equestron on the initiation of translation (Cooper et al. 1973).

The experiment of Fig. 3-1 failed to reveal the preferential synthesis of any early viral polypeptides, those appearing at 3.5 h after infection being in the same proportion as those produced earlier. To determine if poliovirus produced any additional polypeptides late in infection,
Fig. 3-2  The polypeptides synthesized by poliovirus during the infection cycle. Parallel infected cultures were labelled for 45 min starting at the indicated times after infection. Samples containing equal amounts of radioactivity were analysed in 12% gels together with a sample prepared from uninfected cells.
whole cell extracts were examined at times from 2 to 5 h after infection (Fig. 3-2). The only difference readily observed was that the polypeptide designated VP2 was produced at a faster rate at early times in the infection cycle. The rate of protein synthesis later than 5 h after infection was too low to allow satisfactory analysis. Hence, both of these experiments fail to show the presence of any regulatory mechanism controlling the differential production of poliovirus proteins.

(b) The number of poliovirus polypeptides detected in infected cells

The classic report of Summers et al. (1965) first demonstrated fourteen polypeptides synthesized in poliovirus-infected cells. Since then, the only convincing demonstrations of extra polypeptides in infected cells incubated in standard conditions have been by Jacobson et al. (1970) who described the polypeptide NCVPX, and by Taber et al. (1971) who described the polypeptides NCVP1½ and NCVP3a and NCVP3b. The autoradiogram in Fig. 3-2 indicated that more than this number could be resolved by the method used. More definitive experiments were designed to confirm this observation, and perhaps extend it.

A cell culture 3.5 h after infection was labelled with ³⁵S-methionine for 5 min. After a chase period of 2 min, the culture was immediately transferred to 4 volumes of cold PBS and maintained at ice temperature. A culture of uninfected cells was treated in parallel. One portion of the infected cells was then used for the preparation of a cytoplasmic extract by Dounce homogenization. The three samples were then analysed by gel electrophoresis and an autoradiogram prepared (Fig. 3-3).
Fig. 3-3  Autoradiogram of the polypeptides produced in whole infected cells during a pulse of 5 min followed by a chase of 2 min, compared with those found in cytoplasmic extracts of the cells from the same culture, uninfected cells and partially purified virions. Samples were matched approximately for radioactive content and electrophoresed in 12% acrylamide gels. Gels from left to right: uninfected whole cell extract, infected whole cell extract, infected cytoplasmic extract, virus marker.
As previously shown, the heterogeneous distribution of radioactivity seen in the gel from uninfected cells is not apparent in the other gels. Conversely, very few if any of the bands in the gels from infected cells have prominent counterparts in the gel of the uninfected cells, which confirms that host protein synthesis has been effectively suppressed.

Examination of the gel of the whole infected cells in Fig. 3-3 shows 21 radioactive bands clearly discerned, counting only one band in the region of VP3. Since this separation had detected only those polypeptides labelled during a short pulse period, and that other polypeptides were likely to be found after longer chase periods, it became clear that the autoradiographic method was able to allow detection of more poliovirus-specific polypeptides than had been known previously.

Three other aspects apparent in the separations in Fig. 3-3 will be discussed in later sections: the apparent multiple banding of the VP3 region (Chapter 5), the apparent differences between cytoplasmic extracts and whole cell extracts (Section 3.4), and the complex nature of the bands in the VP1 and VP4 regions (Chapter 5).

(c) **A proposed system of nomenclature for poliovirus polypeptides**

The nomenclature of poliovirus polypeptides has already become cumbersome due to lack of flexibility (Jacobson et al. 1970). To enable characterization of newly-detected polypeptides, a new system of nomenclature is proposed which has the advantages of being expandable and also characterizing polypeptides according to their size. Viral capsid proteins
Fig. 3-4  Densitometric tracing of the gel of the whole infected cells in Fig. 3-3. Polypeptides are named as indicated in the text. Electrophoresis is from left to right.
(VP1 to VP4) will continue to be named according to the system proposed by Summers et al. (1965) and the fifth structural polypeptide found in empty capsids will continue to be named VP0 as proposed by Jacobson & Baltimore (1968a). Polypeptides not found in either capsids or empty capsids will be named by the prefix PP (poliovirus polypeptide) and its apparent molecular weight in thousands of daltons. The total number of polypeptides found in various types of experiment in this thesis, together with their former names, is listed in Table 1 found in the Appendix. The values taken as those most nearly representing the molecular weight values of each polypeptide are derived according to the method of Shapiro et al. (1967). The standard methods of calibration and measurement used in the molecular weight determinations are presented in Chapter 4.

Densitometric tracing of a gel autoradiogram provides a useful means for the quantitation of the radioactivity in each band, as well as producing a profile similar to those produced by the former method of scintillation counting. Such a tracing of the infected whole-cell gel in Fig. 3-3 is shown (Fig. 3-4) together with some of the major polypeptides named using the newly-proposed system. Correlation of the major polypeptides in this figure with those described previously was straightforward. Three of the polypeptides from purified virus capsids in Fig. 3-3 showed almost identical electrophoretic mobilities to major bands in the infected whole-cell pattern. Also, the largest, heavily labelled polypeptide seen in the gel from infected cytoplasm seemed to correspond to the polypeptide NCVP1 named by Summers et al. (1965). These observations were confirmed
in later pulse-chase experiments in which the unstable nature of NCVP1 (named PP110) and the concomitant accumulation of radioactivity in the capsid protein regions were demonstrated (Section 3.3). The stable nature of the major polypeptide PP79 in a chase period suggested that it was identical to the polypeptide NCVP2 (Summers et al. 1965; Summers & Maizel, 1968). However, difficulty was encountered in trying to identify which of nine minor polypeptides migrating between PP79 and VP0 corresponded to the three described polypeptides NCVP3, NCVP4 and NCVP5 (Summers et al. 1965). The autoradiographic method used here provided superior resolution to that obtained previously and shows that the broad peaks of radioactivity observed in these regions by Summers et al. (1965) were, in fact, composed of more than one polypeptide species. Hence, six new poliovirus polypeptides are described in this region of gels. That at least several of these polypeptides are virus-coded is confirmed by the fact that they show changes in radioactive content during a chase period—a feature typical of poliovirus polypeptides (Section 3.3).

The only other polypeptide in Fig. 3-4 which did not have an obvious counterpart in the classification of Summers et al. (1965) was PP29 which migrated slightly faster than VP1.

3.3 Proteolytic Cleavage Demonstrated by Pulse-Chase Experiments

Previous demonstrations of extensive proteolysis of labelled polypeptides in poliovirus-infected cells (Summers et al. 1968; Jacobson & Baltimore, 1968b) have employed pulses with either $^{14}$C or $^3$H-labelled amino acids, followed by chases with unlabelled amino acids. The use of high specific activity $^{35}$S-methionine combined with the superior resolution
Poliovirus polypeptides present in infected cells after a pulse of 1 min with $^{35}$S-methionine followed by lengthening chase periods (minutes). Samples containing the equivalent of $7 \times 10^5$ cells were analysed in 12% gels together with an uninfected cell sample labelled for 30 min in parallel, and matched for radioactive content with infected cell samples.
and convenience provided by autoradiography, has allowed shorter pulses to be examined followed by a greater number of chases. The results obtained from these experiments extend the information available about proteolytic cleavage involved in the production of poliovirus proteins.

(a) The fate of polypeptides labelled during a short pulse

A cell culture was incubated for 4 h following infection by poliovirus. $^{35}$S-methionine was added and incubation continued for 1 min. An equal volume of warm culture medium containing excess unlabelled methionine was then added, and immediately an aliquot taken and mixed with 4 times its volume of cold PBS maintained at ice temperature. Aliquots of the parent culture were sampled similarly at times to give chase periods of 1, 2, 5, 12, 30 and 60 min. Samples were prepared for electrophoresis and analysed as shown in Fig. 3-5.

Several previous observations are confirmed by this autoradiogram. The over-all shift of radioactivity during the chase period from higher to lower molecular weight species (Summers & Maizel, 1968) is clearly demonstrated. The unstable nature of the prominent polypeptide PP110 and the subsequent appearance of the capsid polypeptides can be visualized; in contrast, the polypeptide PP79 appears to be stable (Summers & Maizel, 1968).

Much of the radioactivity after a pulse of 1 min is diffusely distributed throughout the gel and presumably represents radioactivity in nascent polypeptides (Butterworth & Rueckert, 1972). This effect diminishes during the initial chase period as more radioactivity accumulates in the higher
molecular weight polypeptides, which accumulation continues up to the 5 min chase period when the largest resolved polypeptides (PP168 and PP155) are maximally labelled. This observation confirms that approximately 10 min is required for a ribosome to complete a single translation of the poliovirus RNA (Taber et al. 1971). The autoradiographic technique is thus able to show both a "chase up" and a "chase down" of radioactivity.

The gel following a 1 min pulse also reveals that some labelled polypeptides can be cleaved to produce completed polypeptides within this time (eg. PP110, PP90, PP66 and PP58). These polypeptides are consequently likely to be the result of single cleavage actions or the product of two cleavages at preferred loci.

More subtle variations can be seen among some of the minor polypeptide species migrating between PP79 and VP0. At least three of these polypeptides (PP73, PP66 and PP58) show a distinct loss in radioactivity during the chase, and another (PP44) shows a gain. Although methionine has been shown to initiate protein translation in poliovirus-infected cells and to be subsequently removed from the nascent polypeptide (Chatterjee, Koch & Weissbach, 1973), no evidence is available to determine which of the polypeptides seen in this experiment contains the initiating $^{35}$S-methionine residue.

(b) Quantitation of the changes in radioactive content during a chase period

In order to put these effects on a quantitative basis, the radioactivity in each band in each of the chased gels (Fig. 3-5) was measured densitometrically. Profiles of each gel similar to that shown in Fig. 3-4 were obtained and
Fig. 3-6  The kinetic behaviour of several polypeptides during a chase period. The percentage of the total gel radioactivity contributed by each polypeptide was calculated from densitometric data at 5 intervals during a chase period, and plotted against the length of the chase.
the areas under each peak measured either by cutting out and weighing the separate pieces, or by tracing on to squared paper. Both methods gave similar results, and the absorbance value for each band was expressed as a percentage of the total absorbance. Preliminary trials with this autoradiogram revealed that the exposure time was such that each band intensity recorded by the densitometer was always proportional to the radioactivity in the gel band.

The proportion of the total radioactivity contributed by each band was plotted against the time of chase to show graphically the changes in each polypeptide. Examples of several such plots are shown in Fig. 3-6. From the series of twenty such curves obtained, the time taken for each unstable polypeptide to lose one-half of its radioactivity was determined from the corresponding graph. Similarly, the half-time of appearance of polypeptides arising at later times during the experiment were estimated. The values for the half times of change for polypeptides showing a change greater than 2% in total radioactivity of the gel during the chase period are shown in Table 2. The two polypeptides PP168 and PP155 were not readily separated by densitometry and are considered as a single entity for this analysis since the kinetics of their behaviour appears to be the same. Portions of the graphs for polypeptides showing a large increase in radioactivity within 2 min of labelling are not considered in this analysis due to the difficulty in accurately quantitating bands in the early part of the chase period.

Fig. 3-6 shows curves representative of the types found for all the poliovirus polypeptides. PP110 and PP58 both decline with simple first order kinetics, while
PP79 and PP29 are stable. The band corresponding to VP1 shows more complex behaviour, with most of its radioactivity appearing quickly and then increasing more slowly. Such behaviour is consistent with more than one species being present, a concept supported by the fact that the VP1 region grows much faster than PP110, the identified precursor of VP1, decays. This point was later confirmed in different electrophoretic separations which allowed the stable polypeptide PP31 to be resolved from VP1 (Chapter 5).

The results in Table 2 show clear differences in the magnitudes and rates of the changes and the times at which they occur. The column showing the times of most rapid change indicates that PP90, PP73 and PP58 all decay soon after commencement of the chase while those that appear during the same time are (VP1 + PP31), VP3 and PP16. Similarly, the cleavage leading to the appearance of VP2 is the last to occur.

The aim of this quantitative analysis was to gain data about the kinetics of the changes in various polypeptides in the hope that they would throw some light on the pathway of cleavage. Unfortunately, many cleavages occurred within 5 min of labelling which complicated the analysis and so additional information is needed to give conclusive findings from this approach. Conclusions which were possible from this data generally supported previous findings. However, three new qualitative observations of interest are shown in this analysis. Firstly, PP29 is shown to be stable showing kinetic behaviour similar to that of PP79. Evidence presented in Chapter 5 suggests that PP29 is distinct from the stable polypeptide PP31 (formerly NCVPX) observed by Jacobson &
<table>
<thead>
<tr>
<th>POLYPEPTIDE</th>
<th>MAGNITUDE AND DIRECTION OF CHANGE (% OF TOTAL RADIOACTIVITY IN GEL)</th>
<th>TIME OF MOST RAPID CHANGE (min. after pulse)</th>
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<td>PP168 + 155</td>
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</tr>
<tr>
<td>PP110</td>
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</tr>
<tr>
<td>PP 90</td>
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<td>PP 66</td>
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</tr>
<tr>
<td>PP 58</td>
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<td>PP 55</td>
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<tr>
<td>PP 44</td>
<td>+ 3.2</td>
<td>13</td>
</tr>
<tr>
<td>PP 40</td>
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<td></td>
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<tr>
<td>PP 38</td>
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<td>VP 2</td>
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<td>VP 3*</td>
<td>+ 15.3</td>
<td>+ 7 }</td>
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<td>PP 20</td>
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<tr>
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<td>VP 4 + PP 8</td>
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<td>+ 15 }</td>
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Table 2. Kinetic behaviour of the poliovirus polypeptides during a chase period. Following densitometric quantitation of the contribution of each polypeptide to the total radioactivity in each of the gels in Fig. 3-5, the magnitude of change which occurred during the chase was derived. From the series of graphs obtained, the half-times of appearance (plus) or decay (minus) for each polypeptide were determined. In each case, the half-time was determined at the time of most rapid change. * The values given for VP3 are the sum of the 3 bands, 2 of which are later shown to be artifacts.
Baltimore (1968b). Secondly, PP73 has a half-life of only 2 min which partially explains why it has not been detected previously. Thirdly, polypeptide PP16 shows unique behaviour for a small polypeptide in that it displays a maximum radioactive content 6 min after commencement of labelling, and then loses at least half of its radioactivity to an unknown smaller molecule.

(c) The absence of detectable proteolysis in uninfected cells

Results with poliovirus-infected cells indicated that the autoradiographic technique employed was eminently suitable for the analysis of subtle changes in the distribution of radioactivity following a short pulse. At the time this work was done, the only data available concerning generalized proteolytic cleavages in the formation of mammalian proteins were inconclusive (Jacobson & Baltimore, 1968b), and so this aspect was re-examined in the following experiment using the currently-available technique. Since then, a recent report (Taber et al. 1973) has provided evidence that about 5% of mammalian proteins may be modified by post-translational cleavages in this way.

One of two parallel cell cultures was infected and incubated for 3.5 h. After labelling both cultures with $^{35}$S-methionine for 2 min, aliquots were immediately chilled (as previously) for the preparation of pulse samples. The remainder of the cultures were chased with medium containing excess unlabelled methionine and aliquots taken after intervals of 1, 5 and 60 min. The results of the electrophoretic analysis of these samples are shown in Fig. 3-7. The samples prepared from the infected cells show the usual
Fig. 3-7 Polypeptides present in uninfected and infected cells after a pulse of 2 min with $^{35}$S-methionine followed by lengthening chase periods (minutes). Samples containing the equivalent of $4.5 \times 10^5$ cells were analysed in 10% gels.
chase pattern of radioactivity into lower molecular weight polypeptides. No such change can be seen in the uninfected samples, which suggests that extensive proteolysis does not occur in the post-translational modification of host cell proteins. However, the small amount of proteolysis claimed by Taber et al. (1973) to occur in host cells is not disputed by this experiment.

(d) The cleavage process at different times during the infection cycle

The only previous investigation of cleavage patterns at different times throughout infection was limited to the examination of the products appearing after a single chase period of 5 min (Summers et al. 1965). Their series of experiments showed that once infection was established the same virus-specific polypeptides were made thereafter. Since the autoradiographic approach was capable of more detailed resolution of cleavage products, it was applied at various times to see if any evidence could be found for the preferential early or late appearance of proteins which may have reflected regulatory functions.

Cells were infected and 1.5 h later, labelled for 1 min with $^{35}$S-methionine and a sample taken for analysis. The remainder of the culture was chased with excess unlabelled methionine and samples taken similarly after further periods of 2, 30 and 60 min. This whole experiment was repeated at 2.5, 3, 4 and 5 h after infection (Fig. 3-8). Cells labelled at 5 h after infection did not show sufficient incorporation of radioactivity to allow analysis. Most of the bands labelled at 1.5 h are of host origin and the first viral proteins can be seen in the 2.5 h samples above the generally
Fig. 3-6 The cleavage process in infected cells at various times after infection. After a pulse of 1 min with $^{35}$S-methionine, samples at the times after infection indicated were chased for lengthening chase periods. Samples containing the equivalent of $7 \times 10^5$ cells were analysed in 12% gels. (a) pulsed samples (b), (c) and (d) chased samples for periods of 2, 30 and 60 min respectively.
diffuse background. Those that can be seen do not appear to
differ qualitatively from those produced at 3 and 4 h after
infection when viral protein synthesis is at a maximum. It
is concluded that the cleavage mechanism is consistent
throughout infection and is not subjected to regulation.

3.4 Apparent Differences between the Polypeptide
Compositions of Whole Infected Cells and Cytoplasmic
Extracts of Infected Cells.

Since poliovirus replicates entirely in the
cytoplasm of cells, it has become accepted dogma that analysis
of the poliovirus proteins in cytoplasmic extracts would
accurately represent the state of the infected cell. To test
this assumption, preliminary experiments in the present work
were done to examine the feasibility of electrophoretic
analysis of whole cells digested with SDS, 2-mercaptoethanol
and urea. When such samples were compared to those made from
a cytoplasmic extract of the same culture, differences were
observed (see Fig. 3-1). The experiments described below
are two of several in which differences were found.

Experiment (1). Uninfected cells were incubated
in parallel to infected cells for 3.5 h. 35S-methionine was
added to both cultures and incubation continued for 5 min
prior to a chase period of 2 min. Both cultures were
immediately chilled by adding to 4 times their volume of PBS
in an ice bath. After washing with cold PBS, one portion of
each pellet was treated immediately with SDS, 2-mercapto-
ethanol and urea, and heated in preparation for electrophor-
esis. The other portions were disrupted in a Dounce homogen-
izer, the nuclei removed by centrifugation, and the super-
natant cytoplasm used to prepare samples for electrophoresis
Fig. 3-9 Comparison of the polypeptides of cytoplasmic extracts with those of whole cells and purified virus. Experiment (i). Samples were prepared as described in the text, matched approximately for radioactive content and analysed in 12% gels. Experiment (ii) Samples were prepared similarly and analysed in 10% gels.
as before. All procedures were done at temperatures lower than 4°C.

Experiment (ii). The procedure of Experiment (i) was repeated exactly, except that the nuclear fractions from both cultures were retained as well after preparation of the cytoplasmic extracts. These portions represented approximately 35% of the total radioactivity incorporated into the cells, and were treated for electrophoresis as above. Since both of the latter samples were very viscous due to the high content of DNA strands, they were frozen and thawed twice in an effort to reduce the viscosity. Both experiments were analysed separately and are depicted in Fig. 3-9 for comparison.

In both experiments, cytoplasmic extracts of infected cells contain reduced amounts of PP168 and PP155. Analysis of the nuclear fraction in the second experiment shows that it appears to contain these two polypeptides in similar proportions to the whole cell extract. This observation excludes the possibility that these two large polypeptides were cleaved during the production of the cytoplasmic extract. They are presumed to remain attached to large membrane fragments which sediment with the nuclei.

The different distribution of PP73 seen in the first experiment is not so pronounced in the second. This polypeptide has a very short half-life in the infected cell (see section 3.2) and so it is possible that in the first experiment, it was cleaved during the production of the cytoplasmic extract. In both experiments, the distribution of PP58 relative to nearby polypeptides seems to have been altered during the production of the cytoplasmic extract.
That it is not cleaved during this process is shown by its abundance in the nuclear extract. Similarly, the polypeptide PP40 appears to be selectively removed from the cytoplasm with the nuclear fraction.

Both experiments show that in whole cell extracts the VP3 region can be resolved into more than one band - an effect that is absent in cytoplasmic extracts. This multiple banding phenomenon of the VP3 polypeptide is examined extensively in Chapter 5, and evidence presented suggesting it is an electrophoretic artifact.

After the completion of this work, a report appeared (Vanden Berghe, 1973) which claimed that two new poliovirus-specific polypeptides were only detected in nucleus-associated material from infected cells. He nominated one of these polypeptides to have a molecular weight of 54,000 daltons, and the other to migrate between VP0 and VP1. In the analysis of the nuclear fraction described here, no unique polypeptide is seen that does not appear in the cytoplasm. However, two polypeptides (PP40 and PP58) in this region do seem to be relatively enriched in the nuclear fraction, and may be the same polypeptides reported by Vanden Berghe.

3.5 Discussion

The detection of exclusively virus-coded polypeptides requires that host protein synthesis is completely inhibited. No single experiment shows conclusively that the proteins being synthesized in poliovirus-infected cells are entirely coded for by viral RNA. However, the likelihood of host protein synthesis persisting in the presence of rapid viral protein synthesis is very low, as suggested by the
following combined evidence of several different approaches.

Firstly, the use of guanidine, which has no effect on host protein synthesis, but does completely suppress viral replication, can inhibit the synthesis of all proteins in poliovirus-infected cells (Holland, 1964). Secondly, the use of an agent to inhibit specifically postulated cleavage enzymes (see Chapter 4) can prevent the appearance of all polypeptides smaller than 80,000 daltons in poliovirus-infected cells. Thirdly, the evidence presented in Section 3.2 on the transition from host to virus-directed synthesis is strong presumptive evidence that the only polypeptides being synthesized by infected cells late in the infection cycle are virus-specific.

The method used here for the detection of poliovirus polypeptides provides superior resolution to that achieved previously. Visual examination of a gel pattern is more convincing than the plot of a radioactivity profile and this has led to the detection of at least six new polypeptides in infected cells all of which are present in small proportions. Figures 3-3 and 3-5 also show that two unstable polypeptides larger than PP110 can be resolved without the use of artificial conditions which have been necessary previously (Cole & Baltimore, 1973). The total number of polypeptides synthesized by poliovirus cannot be observed in a single gel separation since different polypeptides appear at different times during a chase period (Fig. 3-5), and a single set of gel conditions cannot resolve adequately all polypeptides over a wide molecular weight range. This latter point is demonstrated in the following chapter in which the use of more dilute gels detects a further new polypeptide between PP110 and PP90.
(see Fig. 4-8), and again in Chapter 5 where the omission of urea from the gels leads to the resolution of PP31 and PP8 from VP1 and VP4 respectively (see Fig. 5-1). Neglecting the fact that VP3 appears to be resolved into 3 bands (see Chapter 5 for discussion) inspection of all the gels in Fig. 3-5 reveals a total of 23 polypeptides. Addition of the three further polypeptides to be demonstrated later gives a total of 26 virus-specific polypeptides found in infected cells incubated in standard conditions.

Attempts were made to derive information about the poliovirus cleavage pathway by studying the kinetic behaviour of each polypeptide after a short pulse period (Section 3.3 b). Success was achieved in establishing values for the half-lives of those polypeptides cleaved during a chase period, and values for the half-time of appearance of stable products. During this work, a report appeared (Butterworth & Rueckert, 1972) in which a kinetic approach was used to study the protein cleavage of another picornavirus, encephalomyocarditis (EMC), following a short pulse. At least two basic facts allowed this analysis to proceed. Firstly, an intermediate in the breakdown of a large precursor molecule to the capsid proteins was known, and secondly, the precursor molecules of the largest stable polypeptide in the EMC system (corresponding to PP79 in the poliovirus system) were present in the infected cells after a short pulse. The number of polypeptides analysed by Butterworth & Rueckert (1972) and found to fit into their scheme was eleven. A further six polypeptides were cleavage products from unknown precursors. Although the kinetic approach used provided neat explanations for the cleavages of two large precursors, the full cleavage
mechanism was still far from being solved. Since the poli-
virus cleavage mechanism lacked the useful facts of the EMC system and seemed to be more complex, a similar detailed approach did not appear justified. However, the values for the half-times of appearance and decay for the various polypeptides determined in this chapter were used in attempts to formulate the possible cleavage sequence of poliovirus polyprotein described in Chapter 6.

Experiments showing the presence of the same cleavage mechanism throughout infection (Fig. 3-8) and the absence of detectable proteolytic cleavage in uninfected cells (Fig. 3-7) demonstrated the uniqueness and consistency of the poliovirus cleavage mechanism. The former experiment showed as well that the cleavage mechanism is not subjected to any form of regulation during the infection cycle, which indicates that the cleavage specificity is governed only by the intrinsic properties of the poliovirus proteins. Differences were observed between the polypeptide compositions of infected whole cells and cytoplasmic extracts of infected cells (Fig. 3-9). In all, four such experiments were done to clarify this point. In neither of the two experiments not shown here was any difference found that is not revealed in the two analyses shown. Differences that were observed were less pronounced than those shown in Fig. 3-9 and hence this type of experiment produced variable results. It is concluded that cytoplasmic extracts differ marginally from whole cell extracts, in both qualitative and quantitative aspects. The polypeptides PP168 and PP155 have not previously been reported in infected cells grown in the absence of inhibition of cleavage, and the most likely reason for this is that cyto-
plasmic fractionations invariably used in the past have lost these polypeptides into the nuclear fractions.

This work fails to substantiate the report of Vanden Berghe (1973) of new polypeptides present in the nuclear fractions of poliovirus-infected cells. Nevertheless, doubt is raised about cytoplasmic extracts being truly representative of the whole cell situation. The findings reported here together with those of Vanden Berghe suggest that caution should be exercised in the interpretation of results using cytoplasmic extracts.

3.6 Summary

The application of the technique of autoradiography of acrylamide gels to the study of poliovirus-infected cells has been demonstrated. This technique has allowed the detection of 26 virus-specific polypeptides in the normal infected cell, a value that considerably exceeds the number of such polypeptides known previously. The kinetic behaviour of these polypeptides following a short pulse was quantitated and values were obtained for many for the respective half-times of appearance and decay. Considerable variations in these values were found which gave some new insights into the cleavage pathway of poliovirus proteins.

This study confirmed previous observations that, in contrast to other animal virus systems, no mechanisms for the differential regulation of the production of poliovirus polypeptides could be found.

Minor qualitative differences were shown in the polypeptide compositions of whole cell and cytoplasmic extracts of infected cells. It was concluded that for the analysis of proteins, whole cell extracts represented more closely the status of the infected cell.
CHAPTER FOUR

THE EFFECTS OF MODIFIED CULTURAL CONDITIONS ON THE
POLYPEPTIDES OF POLIOVIRUS-INFECTED CELLS

4.1 Introduction

4.2 The Screening of Various Amino Acid Analogues for
Effects on Proteolytic Cleavage
   (a) The effects of various amino acid analogues
       compared with that of p-fluorophenylalanine
   (b) The effect of L-canavanine sulphate on RNA
       synthesis in infected cells

4.3 The Screening of Various Inhibitors of Proteolytic
Enzymes for Effects on Proteolytic Cleavage

4.4 The Effects of Temperature on Proteolytic Cleavage

4.5 A Comparison of the Very Large Poliovirus Polypeptides
Induced by Three Different Methods

4.6 The Molecular Weights of Poliovirus Polypeptides
   (a) Measurement of high molecular weight
       poliovirus polypeptides
   (b) Measurement of low molecular weight
       poliovirus polypeptides
   (c) Discussion

4.7 Summary
4.1 Introduction

Since the demonstration of extensive proteolysis in the production of poliovirus proteins, attempts have been made to demonstrate larger precursor molecules in infected cells. Alteration of the cleavage mechanism using amino acid analogues (Jacobson & Baltimore, 1968b; Jacobson et al. 1970) was successful in the demonstration of two peaks of radioactivity with molecular weights higher than 110,000 daltons, one of which was postulated to be the primary translation product of poliovirus RNA. Subsequently, inhibitors of proteolytic enzymes (Jacobson et al. 1970) and the use of higher temperatures (Baltimore, 1971) have both allowed the accumulation of large polypeptides.

In this chapter, the convenience of the autoradiographic technique for the analysis of acrylamide gel separations is used to screen a much wider range of cultural conditions for altered cleavage effects. Large precursor polypeptides accumulating under different conditions are compared and their molecular weights estimated.

4.2 The Screening of Various Amino Acid Analogues for Effects on Proteolytic Cleavage

(a) The effects of various amino acid analogues compared with that of p-fluorophenylalanine

A suite of amino acid analogues was gathered which covered 7 of the common amino acids and for which there was some likelihood of incorporation during protein synthesis. Concentrated solutions of each analogue were added to infected cells incubated in the Eagle's medium lacking added amino acids. Five minutes later, $^{35}$S-methionine was added for 10 min followed by a chase with Eagle's complete medium. Samples
Fig. 4-1 The analysis in 10% gels of poliovirus polypeptides produced in the presence of amino acid analogues. At 3.5 h after infection, solutions of each analogue were added to separate cultures for 5 min to give a final concentration in each of 3 mM. Cultures were chased for 2 min and 60 min following a pulse of $^{35}$S-methionine for 10 min. One control culture did not have any additions. Samples in pairs represent polypeptides labelled by a pulse and a long chase alternately. (a) control, (b) DL-4-fluorotryptophan, (c) DL-6-fluorotryptophan, (d) allyl-DL-glycine, (e) DL-p-fluorophenylalanine, (f) 4-aza-leucine, (g) L-3-aminotyrosine, (h) DL-β-phenyl-lactic acid, (i) DL-norvaline.
for electrophoresis were taken after 10 and 60 min. The polypeptides produced in the presence of 8 analogues are shown in the autoradiogram in Fig. 4-1, and the effects of each can be compared with that of an analogue of known effect, DL-p-fluorophenylalanine. The inhibiting effect of the latter on cleavage can be most clearly seen in the region of the major capsid polypeptides where all three are appreciably reduced. Analogues producing an effect similar to that of p-fluorophenylalanine are DL-4- and DL-6-fluorotryptophan, while others show little inhibitory action on cleavage in this analysis. Allyl-DL-glycine, DL-norvaline and DL-ß-phenyl-lactic acid are not known to be incorporated into proteins but were included in this experiment as controls. Another twelve amino acid analogues were screened in a similar way and the results summarized in Table 3. Those producing an effect comparable with that of DL-p-fluorophenylalanine are listed as positive, those producing no apparent effect are listed as negative.

Fig. 4-2 shows several analogues compared for their effect on cleavage during a labelling period of 1 h. Samples were analysed in gels without added urea in order to separate better the polypeptides in the VP0 -VP1 region (see Chapter 5). The analogues DL-ß-phenylserine and L-canavanine sulphate are shown to have striking effects which lead to the accumulation of considerable radioactivity in very high molecular weight polypeptides. Hence, single amino acids can lead to the accumulation of very large poliovirus precursors and this observation contrasts with the statement by Jacobson et al. (1970) to the contrary. Resolution of the large polypeptides induced by these analogues is better
Fig. 4-2 The analysis in 10% acrylamide gels (without added urea) of poliovirus polypeptides produced in the presence of amino acid analogues. At 3.5 h after infection, solutions of each analogue were added to separate cultures for 5 min to give a final concentration of analogue in each of 3 mM. $^{35}$S-methionine was added and samples prepared for electrophoresis 1 h later. (a) purified virus, (b) culture without added analogues, (c) DL-$p$-fluorophenylalanine, (d) DL-$\beta$-phenylserine, (e) L-canavanine sulphate, (f) 3,4-dehydro-DL-proline, (g) L-azetidine-2-carboxylic acid, (h) DL-1,2,4-triazole-3-alanine.
demonstrated later in Fig. 4-8. Other analogues have much less dramatic effects, for example the only effect of the two analogues of proline in Fig. 4-2 was to prevent the appearance of VP2. From numerous other experiments in which the cleavage mechanism was disturbed, it was concluded that the cleavage producing VP2 was the one most sensitive to inhibition. This is plausible as it is the final cleavage of protein prior to completion of the virion (Jacobson & Baltimore, 1968a).

Table 3 shows that the analogues effective in the gross alteration of the cleavage mechanism are with the exception of L-canavanine, derivatives of amino acids with aromatic side chains. This observation implicates arginine as having a key role in the determination of either the specificity site for postulated cleavage enzyme(s), or the conformation of the poliovirus precursor proteins. Phenylalanine probably has a similar role as several of its analogues are very active in the prevention of cleavage. This approach to the problem of specificity of cleavage lacks breadth because many of the amino acids have no known analogues that are incorporated into protein. Hence, analogues of only a few amino acids could be screened in this way.

Several analogues effective in these experiments (eg. β-phenylserine, β-thienylalanine, 4- and 6-fluorotryptophan) were not known previously to be incorporated into eucaryotic proteins. That they do have an effect on the post-translational modification of poliovirus polypeptides is strong presumptive evidence for their utilization and incorporation into mammalian cell proteins.
<table>
<thead>
<tr>
<th>AMINO ACID</th>
<th>ANALOGUE</th>
<th>INCORPORATION INTO EUCARYOTIC PROTEINS</th>
<th>EFFECT ON CLEAVAGE OF POLIOVIRUS POLYPEPTIDES</th>
</tr>
</thead>
<tbody>
<tr>
<td>Arginine</td>
<td>L-Canavanine sulphate</td>
<td>+ (a)</td>
<td>Strongly positive</td>
</tr>
<tr>
<td>Histidine</td>
<td>1,2,4-Triazole-3-alanine</td>
<td>Unknown</td>
<td>Negative</td>
</tr>
<tr>
<td>Leucine</td>
<td>4-Azaleucine</td>
<td>Unknown</td>
<td>Negative</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>DL-p-Fluorophenylalanine</td>
<td>+ (c)</td>
<td>Positive</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>DL-o-Fluorophenylalanine</td>
<td>+ (c)</td>
<td>Positive</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>DL-m-Fluorophenylalanine</td>
<td></td>
<td>Positive</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>DL-B-Phenylserine</td>
<td>Unknown</td>
<td>Strongly positive</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8-2-Thienylalanine</td>
<td>Unknown</td>
<td>Positive</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>8-2-Thienylserine</td>
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<td>Negative</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>DL-3,4-Dihydroxyphenylalanine</td>
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<td>Negative</td>
</tr>
<tr>
<td>Proline</td>
<td>3,4-Dehydro-DL-proline</td>
<td>+ (b)</td>
<td>Weakly positive</td>
</tr>
<tr>
<td>Proline</td>
<td>L-Azetidine-2-carboxylic acid</td>
<td></td>
<td>Weakly positive</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>DL-7-Azatryptophan</td>
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<td>Negative</td>
</tr>
<tr>
<td>Tryptophan</td>
<td>DL-4-Fluorotryptophan</td>
<td>Unknown</td>
<td>Positive</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>p-Amino-DL-phenylalanine</td>
<td>Unknown</td>
<td>Negative</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>L-3-Aminotyrosine</td>
<td>Unknown</td>
<td>Negative</td>
</tr>
</tbody>
</table>

Table 3. The amino acid analogues tested for their ability to interfere with the cleavage of poliovirus polypeptides. Analogues whose only apparent effect on cleavage was to prevent the appearance of VP2 are listed as weakly positive. References giving evidence for the incorporation of amino acid analogues into eucaryotic proteins are (a) Krause et al. (1959) (b) Fowden, Neale & Tristram (1963) (c) Vaughan & Steinberg (1960).
The potent effect of canavanine on cleavage is evident with the absence of virtually all polypeptides smaller than about 40,000 daltons including PP31 (Fig. 4-2). Jacobson et al. (1970) indicated that this polypeptide (formerly named NCVPX) continues to be generated in the presence of p-fluorophenylalanine, and indirect evidence implicates it in having a functional role in the synthesis of viral RNA. Its absence in cells in the presence of canavanine formed the basis for the experiments in the next section.

(b) The effect of L-canavanine sulphate on RNA synthesis in infected cells

The genetic analysis of poliovirus using temperature sensitive mutants (Cooper et al. 1971) has revealed that two of the three primary gene functions of poliovirus RNA are concerned with RNA synthesis and map together towards the 3' end of the genetic message. Experiments with the drug pactamycin have allowed an ordering of the principal polypeptides from the 5' end of the poliovirus RNA (Taber et al. 1971; Summers & Maizel, 1971). The order obtained was PP110, PP31, PP79 (5' → 3'). The combined results of these two genetic analyses suggested that PP31 is specified by a region concerned with RNA synthesis and may indeed have such a function. Experiments were designed to test this possibility as this polypeptide is not generated in the presence of L-canavanine. Since p-fluorophenylalanine does not effect either the generation of PP31 (Jacobson & Baltimore, 1968b) or the synthesis of infectious poliovirus RNA (Levintow et al. 1962), it was included as a control analogue.
Fig. 4-3 The incorporation of $^{32}$P into acid-insoluble material from the four cultures described in the text. Panel B represents acid-insoluble radioactivity in aliquots of each culture. Panel A represents the products of the cultures analysed in 15 - 30% sucrose-SDS gradients. (a) uninfected control, (b) infected control, (c) p-fluorophenylalanine-treated, (d) L-canavanine-treated.

- total radioactivity in 50 μl of each fraction.
- acid-insoluble radioactivity resistant to ribonuclease in 50 μl of each fraction. Arrows indicate the sedimentation of 16 S and 28 S ribosomal RNA as indicated by optical density traces.
Preliminary results showed that for cells incubated in Eagle’s medium without added amino acids, infection had to proceed for 1 h in the absence of analogues in order for infection to be established. The minimum concentration of L-canavanine found to cause the prevention of PP31 when added 1 h after infection was found to be 90 μM. A similar active concentration of DL-p-fluorophenylalanine was added to control cultures which allowed the appearance of PP31 but reduced the amounts of capsid polypeptides produced.

Three cultures of infected cells each containing 9 x 10⁷ cells were incubated in Eagle’s medium (lacking added amino acids and phosphates) for 1 h in parallel to another culture which was subjected to a dummy infection. Two of the infected cultures were treated with either DL-p-fluorophenylalanine (180 μM) or L-canavanine sulphate (90 μM). Incubation was continued for a further 2 h after which each culture was split into two portions, one of which was treated with ³²PO₄ (6 μCi/ml) for a further 1.5 h. The uptake of radioactivity into acid-insoluble material was followed. One-half of the product from each labelled culture was analysed on a sucrose - SDS gradient as described in Chapter 2. The other half was treated with pancreatic ribonuclease (10 μg/ml) and analysed similarly (Fig. 4-3).

At 4 h after infection, the remaining portions of each culture were treated with ³⁵S-methionine and the incorporation into acid-insoluble material followed 10 min and 60 min later. Cells from these cultures were then analysed by gel electrophoresis (Fig. 4-4).

The rate of incorporation of ³²PO₄ by all four cultures was similar. When the products were analysed on
The incorporation of $^{35}$S-methionine into acid-insoluble material from the four cultures described in the text. Panel A represents acid-insoluble radioactivity in 10 μl volumes of each culture at the times shown. Panel B represents the polypeptides produced in the cultures analysed in 10% gels without urea. (a) uninfected, (b) infected control, (c) p-fluorophenylalanine-treated, (d) L-canavanine-treated.
sucrose SDS gradients differences were obvious. Only the infected and the fluorophenylalanine-treated cultures showed a peak sedimenting at 35 S which corresponded to viral RNA and a minor peak of ribonuclease-resistant material corresponding to the replicative intermediate (Baltimore, Becker & Darnell, 1964). It is clear from this experiment that canavanine has the capacity to severely restrict the synthesis of poliovirus-specific RNA in infected cells. Since $^{32}$P0$_4$ was incorporated into both uninfected and canavanine-treated cultures, it must be present either within the nucleus or in low molecular weight species at the tops of the gradients. That the canavanine-treated culture did allow infection to proceed is shown in Fig. 4-4. All of the low molecular weight polypeptides synthesized in the presence of canavanine appear to be host-specific. However, at least 3 prominent bands in higher molecular weight regions (70,000 - 100,000 daltons) appear to be virus induced, although one of them (at least) does not correspond to a normal poliovirus polypeptide. This polypeptide pattern differs from that produced when canavanine is added only a short time before labelling (Fig. 4-2). Explanation of this phenomenon may be that canavanine induces the formation of a defective RNA polymerase which does not permit the transcription of authentic viral messenger RNA. Evidence that RNA synthesis is defective in these conditions is shown by the fact that the incorporation of $^{35}$S-methionine in the presence of canavanine is reduced by about one-half (Fig. 4-4). This could be explained by a lack of suitable messenger RNA's, as control experiments showed that low levels of canavanine had little effect on uninfected cell protein synthesis within 6 h.
The experiments described in this section were planned to clarify any role the polypeptide PP31 had in the synthesis of viral RNA. The results were complicated by the fact that low levels of canavanine used from 1 h after infection caused the appearance later of a completely different polypeptide pattern in infected cells. This unexpected result is therefore reported, although no conclusions can be made concerning the original hypothesis. Canavanine certainly interferes with RNA synthesis in infected cells, but in a manner more complex than was anticipated. The results suggested that any polymerases made in the presence of canavanine were probably defective, and so investigations using this approach were pursued no further.

4.3 The Screening of Various Inhibitors of Proteolytic Enzymes for Effects on Proteolytic Cleavage

Evidence implicating enzymes similar to serine esterases in the cleavage of large poliovirus polypeptides was first presented by Jacobson et al. (1970) who showed that di-isopropyl fluorophosphonate could cause delays in the cleavage of poliovirus polypeptides. To try to get more specific information about the proteases involved, several other compounds known to have narrower specificities of inhibition for such enzymes were screened for effectiveness.

Infected cells were incubated for 3.5 h prior to the addition of various serine esterase inhibitors dissolved in dimethyl-sulphoxide at 100 times their final concentration. Replicate 5 ml cultures containing 12.5 x 10⁶ cells were treated with 50 μl of each inhibitor. Five minutes later, ³⁵S-methionine was added and incubation continued for 5 min, prior to the addition of excess
Fig. 4-5 The poliovirus polypeptides synthesized in the presence of various inhibitors of proteolytic enzymes and analysed in 10% gels. The left hand gel of each pair represents a 5 min pulse and 2 min chase, and the right hand gel, a 5 min pulse and 60 min chase. (a) control, (b) dimethyl sulphoxide 1%, (c) tosyl-L-lysine chloromethane 0.32 mM, (d) phenylmethanesulphonyl fluoride 1 mM, (e) toluenesulphonyl fluoride 1 mM, (f) diphenyl carbamyl chloride 0.86 mM, (g) phenyl boronic acid 2.4 mM.
unlabelled methionine. Samples of each culture were taken for analysis 2 min and 60 min later (Fig. 4-5). One culture of cells was treated with dimethyl sulphoxide alone to show it had no effect on the pattern of polypeptides produced (gel b). Tosyl-L-lysyl chloromethane (TLCK), a specific inhibitor of trypsin (Schroeder & Shaw, 1971), and diphenyl carbamyl chloride (DCC), a specific inhibitor of chymotrypsin (Erlanger & Cohen, 1963), both allowed the accumulation of large poliovirus precursors and prevented the appearance of the capsid polypeptides. The three other compounds had no apparent effects at the concentrations used. Other similar experiments showed that tosyl-L-phenylalanyl chloromethane (TPCK), a specific inhibitor of chymotrypsin, was a potent inhibitor of cleavage. At 0.25 mM, TPCK could prevent the appearance of all polypeptides smaller than about 80,000 daltons (Fig. 4-6). This result implies that if poliovirus RNA has more than one site for the initiation of protein synthesis, any additional uncleaved translation product must be at least this size.

These experiments suggest that proteolytic enzymes are responsible for the extensive proteolysis occurring during the synthesis of poliovirus polypeptides. Since TPCK and TLCK have such specific activities against chymotrypsin and trypsin (Shaw, 1967), it seems that at least two different proteolytic specificities are involved in the cleavage of poliovirus precursors. A later experiment shows the difference in activity of these two compounds (see Fig. 4-8). TPCK (but not TLCK) restricts cleavage(s) of the polypeptide PP155, whereas the reverse is true for PP125. Since other polypeptides are freely generated in the presence
Fig. 4-6 The poliovirus polypeptides synthesized in the presence of 0.25 mM-tosyl-L-phenylalanyl chloromethane and analysed in 11% gels. Experimental procedures were the same as for Fig. 4-5. The left hand gel of each pair represents a 5 min pulse and 2 min chase, the right hand gel, a 5 min pulse and 60 min chase.
of both compounds, it appears likely that other proteolytic activities are still operative that are not sensitive to these inhibitors at the concentrations used. DCC also has a specific inhibitory action against chymotrypsin, and it is tempting to implicate this or related proteases as having functions in the proteolysis of poliovirus polypeptides. However, these compounds are also non-specific alkylating agents and may react with the proteins themselves, or with the cell machinery to prevent normal proteolysis. Such side reactions no doubt contribute to the cell toxicity exhibited by these compounds.

During the course of this work, two reports appeared which supported, in part, data presented here. Summers et al. (1972) showed the inhibitory nature of TPCK and one of its optical isomers on the cleavage of poliovirus polypeptides. TLCK was used at lower concentrations than in the present work, and the effect was correspondingly reduced. Korant (1972) showed that the effectiveness of protease inhibitors depended on the cell line used. His work failed to show any effect of TPCK in poliovirus-infected HeLa cells and so doubt is cast on the validity of his other findings.

It was surprising that the inhibitors of serine esterases phenylmethanesulphonyl fluoride and toluenesulphonyl fluoride were without effect in this system. Both of these inhibitors have been shown to be active in vivo against proteases of Escherichia coli (Goldberg, 1971), and so were expected to enter mammalian cells and act similarly.

4.4 The Effects of Temperature on Proteolytic Cleavage

Increased temperature was shown to be able to prevent the normal cleavage of poliovirus precursor proteins
Fig. 4-7  The polypeptides of poliovirus-infected cells incubated at different temperatures. (i) Polypeptides labelled at 43°C and analysed in 10% gels (a) 4.5 min pulse label at 43°C, (b) 4.5 min pulse and 60 min chase at 43°C, (c) 4.5 min pulse at 43°C and 60 min chase at 37°C, (d) virus marker. (ii) Polypeptides labelled at 33°C and analysed in 11% gels (e) pulse label at 33°C for 3 min, (f) to (h) 3 min pulse and chases at 33°C of 4 min, 10 min and 60 min respectively.
In this section, Baltimore's experiment was repeated, and the cleavage of poliovirus proteins at temperatures lower than $37^\circ C$ was investigated.

Infected cells were incubated at $37^\circ C$ for 3.5 h and then at $43^\circ C$ for 5 min. $^{35}S$-methionine was added for 4.5 min followed by excess unlabelled methionine for 2 min. One portion of the culture was immediately chilled, one portion left at $43^\circ C$ and a third transferred to $37^\circ C$ for a further 60 min. The polypeptides synthesized in these cultures are shown in Fig. 4-7.

As reported by Baltimore (1971), incubation at $43^\circ C$ causes the accumulation of large polypeptides which are not cleaved after a long chase. However, continued incubation of such labelled cells at $37^\circ C$ was found to cause the disappearance of large precursors and the accumulation of smaller capsid proteins, a result which contrasted with the observations of Baltimore. This result was reproducible, and the reason for the discrepancy is unknown. When cells were labelled at $43^\circ C$ some cleavages were totally prevented, which reduced the number of functional cleavage loci in the poliovirus polyprotein. At least one of these loci regained accessibility to proteases at $37^\circ C$, and so this system appeared to be a useful model on which the determination of the cleavage pathway might be attempted.

When similar experiments were done at $33^\circ C$ to look for further abnormal cleavage patterns, no such defects could be found (Fig. 4-7). After a pulse of 3 min, chase samples were taken at 4, 10 and 60 min. The patterns found were similar to those obtained at $37^\circ C$. It is concluded that
Fig 4-8: Poliovirus polypeptides synthesized in varying cultural conditions and analysed in parallel in 6% gels. (a) control infected cells, (b) 3 mM p-fluorophenylalanine, (c) 3 mM DL-β-phenylserine, (d) 3 mM L-canavanine sulphate, (e) virus marker, (f) 0.12 mM-TPCK, (g) 0.32 mM-TLCK, (h) 0.43 mM-DCC, (i) incubated at 43°C.
poliovirus precursors do not fold at 33°C in a fashion inaccessible to the proteases, and that the proteases are equally active at this temperature.

4.5 Comparison of the Poliovirus Precursors Produced by Three Different Methods

The results of the three previous sections have shown that large precursor polypeptides can be induced by different sets of cultural conditions. In order to compare such large polypeptides for identity and also for quantity, electrophoresis was done in parallel in dilute acrylamide gels.

Infected cells were incubated for 3.5 h and divided into eight separate cultures. One acted as a control and others were (i) treated with amino acid analogues at final concentrations of 3 mM, (ii) treated with inhibitors of proteolytic enzymes, or (iii) incubated at 43°C. Five minutes later all cultures were labelled for 1 h with 35S-methionine and then samples analysed as shown in Fig. 4-8.

A very large polypeptide is present following treatment with β-phenylserine, canavanine, TPCK, TLCK, and high temperature. This polypeptide has a similar electrophoretic mobility in all gels and is believed to be the complete translation product of poliovirus RNA, named PP210. The next largest polypeptides common to many of the gels are PP168 and PP155 which correspond with the polypeptides able to be detected in infected cells in a short pulse (see Chapter 3). Other large precursor polypeptides are not found under all conditions tested. TPCK and TLCK induce different precursor molecules, in different relative proportions. This observation supports the idea that these two compounds inhibit
Fig. 4-9  Poliovirus polypeptides synthesized during short pulses under different cultural conditions and analysed in 7.5% gels. (a) chases of 2 min and 60 min following a 2 min pulse, (b) chases of 2, 5 and 60 min following a 2 min pulse in the presence of p-fluorophenylalanine plus L-canavanine, (c) chases of 2 min and 60 min following a 2 min pulse in the presence of TPCK.
at least two different proteolytic specificities. The effect of DCC in this experiment was not pronounced; several experiments showed this compound to be variable in its effect.

Both β-phenylserine and canavanine produce almost identical polypeptide patterns. This is an unexpected finding since one is an analogue of arginine and the other is an analogue of phenylalanine - two dissimilar amino acids. This effect on the total structure of the poliovirus polyprotein must be such that the same cleavage loci are rendered inaccessible to the usual proteases.

The dilute gels in this figure are better able to resolve high molecular weight polypeptides which leads to the detection in control gels of an additional polypeptide migrating between PP110 and PP79. The larger of these two polypeptides is named PP96 while the other, PP90, corresponds to the polypeptide formerly known as NCVPl₂ (Jacobson et al. 1970).

Even more large polypeptide precursors can be detected if modified cultures are analysed after a short pulse only. Infected cells were divided into three cultures, one being a control. To another was added a mixture of DL-p-fluorophenylalanine and L-canavanine sulphate each at 3 mM, and to the third, 0.12 mM-TPCK. Five minutes later, ³⁵S-methionine was added for 2 min and then chased with unlabelled methionine for 2 min and 60 min. The sample containing analogues also had a sample chased for 5 min (Fig. 4-9). An additional 3 polypeptides can be seen migrating between PP210 and PP168 in both gels (b) and (c), and are named PP200, PP185 and PP176. This brings the total number of bands detected in poliovirus-infected cells under all conditions
tested to 34. Not included in this number is a diffuse band of radioactivity seen only during a short pulse and migrating slightly slower than PP110 (see Fig. 4-9). All efforts to resolve this region into 1 or more discrete bands have failed.

Summarizing the results presented in this section (4.5), it can be concluded that in various cultural conditions large poliovirus polypeptides accumulate some of which appear to have identical relative mobilities. The appearance of some polypeptides (eg. PP110, PP90, PP79) is not prevented by any of the cultural conditions tested. Also, the appearance of the polypeptides PP168 and PP155 is common to many of the cultures. These observations suggest that all of these larger polypeptides must be the products of cleavages at preferred loci in the poliovirus polyprotein; cleavages at other loci are more sensitive to external interfering influences. The observation that at least 34 different polypeptides can be detected in poliovirus-infected cells in all tested conditions implies that a minimum of seven cleavage loci (which can allow a possible 35 products) must be present in the polyprotein. This figure is likely to be conservative, as the cleavage of VP0 to (VP2 + VP4) occurs at least 30 min after translation, and so theoretically possible products resulting from cleavage at this locus are probably not detected.

4.6 The Molecular Weights of Poliovirus Polypeptides

The only method available at present for the determination of the molecular weights of the majority of poliovirus polypeptides is to measure their relative electrophoretic mobilities in SDS-containing acrylamide gels
(Shapiro et al. 1967). This method is not infallible and some proteins behave irregularly (Dunker & Rueckert, 1969). Also, the relationship between mobility and molecular weight over a wide range deviates from linearity as the gel concentration is altered (Weber & Osborn, 1969). Variations in experimental conditions together with the use of different standards has led to different values being published for poliovirus polypeptides (Maizel & Summers, 1968; Jacobson et al. 1970).

In this thesis, an effort is made to overcome some of the problems of the method, and to determine more accurately molecular weights of poliovirus polypeptides. Needless to say, the system of nomenclature suggested in the previous chapter is dependent on the accuracy and reproducibility of these values.

Since dilute gels are necessary to resolve adequately very large polypeptides, molecular weights for these species are determined separately from those for smaller polypeptides. For reasons to be given in detail in Chapter 5, all molecular weight determinations were done in gels containing 5 M-urea as well as SDS. Also, since preliminary results indicated that the size of the sample load could affect relative mobility, all measurements were done with standards and unknowns treated identically and electrophoresed in the same gel. All standard markers were monomeric polypeptides which had been fully reduced to ensure complete denaturation. These basic principles were adopted in an effort to eliminate some sources of variation.

(a) Measurement of high molecular weight poliovirus polypeptides

Suitable polypeptides for use as standard molec-
Electrophoresis of $^{125}$I-labelled standard polypeptides with $^{35}$S-labelled poliovirus polypeptides in 6% gels. Gels in group (ii) contained no urea. 
(a) $^{125}$I-labelled myosin and haemocyanin, (b) $^{125}$I-labelled human albumin, (c) mixture of all three standards, (d) mixture of all three standards plus poliovirus polypeptides labelled in the presence of 0.12 mM-TPCK, (e) poliovirus polypeptides labelled in the presence of 0.12 mM-TPCK, (f) poliovirus polypeptides labelled in the absence of TPCK.
ular weight markers in this region are rare. Rabbit skeletal muscle myosin contains two similar polypeptide chains which can be purified and their molecular weights measured by ultracentrifugation. Various laboratories disagree on the exact value of the molecular weights of these chains (Lowey et al. 1969; Gazith, Himmelfarb & Harrington, 1970) with estimates ranging from 194,000 to 220,000 daltons. A value determined in a careful study by Gershman et al. (1969) of 212,000 ± 5,000 daltons is used as a standard in this work. Other well-characterized proteins available with large polypeptide chains were crayfish haemocyanin (86,000; Parish & Marchalonis, 1970) and human albumin (66,000).

In order to have internal radioactive markers for the estimation of the molecular weights of poliovirus polypeptides, the three standard proteins were iodinated with $^{125}$I (see Chapter 2). Preliminary results showed that iodination did not alter the relative mobilities of any of the standards, and that radioactive bands located by autoradiography co-electrophoresed exactly with bands of protein detected by staining. These three markers were then electrophoresed in the same gel as poliovirus polypeptides labelled with $^{35}$S-methionine in the presence of TPCK. Electrophoresis was done in parallel in two sets of gels, only one of which contained urea (Fig. 4-10). In the presence of urea, the largest poliovirus polypeptide migrates slightly faster than the iodinated myosin marker. In the absence of urea it appears to migrate slower than myosin. The determination of the exact location of the $^{125}$I label in gel (d) could be achieved by differential detection of γ-emissions. This technique involved the use of two-sided X-ray film in
Fig. 4-11  (i) Electrophoretic mobilities in 6% gels containing 5 M-urea of the three standard polypeptides from gel (d) in Fig. 4-10 plotted against their molecular weights. The migration positions of five prominent poliovirus polypeptides are shown. (ii) Electrophoretic mobilities in 10% gels containing 5 M-urea of four standard polypeptides plotted against their molecular weights. The migration positions of the five polypeptides found in poliovirus capsid structures are shown. (TMV = tobacco mosaic virus protein).
which the emulsion on the exposed side only was activated by the \( \beta \)-emissions of \( ^{35}\text{S} \), which were too soft to pass through the film. Examination of the reverse side of the film in oblique light showed where the higher energy \( \gamma \)-emissions of \( ^{125}\text{I} \) had passed through the film and so activated the emulsion on both sides. This method showed that in the absence of urea, myosin co-migrated exactly with the largest poliovirus polypeptide. Hence, in neither of these separations is the electrophoretic mobility of the largest poliovirus polypeptide any slower than that of myosin. This observation contrasts with the observation of Summers et al. (1972) and illustrates the unreliability introduced by different sample loadings when molecular weight measurements are determined in separate gels. Since larger amounts of \( ^{125}\text{I} \)-labelled haemocyanin and human albumin were used in this experiment, no doubt exists as to their location; haemocyanin migrates slightly faster than PP90 and human albumin slightly slower than PP58 in both gel conditions.

The three iodinated markers were used to construct a standard line by the method of Shapiro et al. (1967) from which the values for the principal poliovirus polypeptides could be determined (Fig. 4-11). From five such experiments in which the co-ordinates of the marker proteins were reproducibly colinear, average molecular weights were determined for four of the polypeptides which led to the designations PP210, PP110, PP90 and PP79. The migration position of PP31 (having an apparent molecular weight of 30,000 daltons in this gel) is included to show that linearity of the plot almost extends to include polypeptides of this size. Values for other large poliovirus polypeptides were
then determined relative to these values, and are recorded in Table 1 found in the Appendix.

(b) Measurement of low molecular weight poliovirus polypeptides

Internal radioactive markers are unnecessary for the measurement of the sizes of capsid polypeptides as the samples loaded on to gels are low enough to permit staining of gels for protein. Consequently, standard proteins were electrophoresed in the same gel as an artificial mixture of $^{35}$S-labelled top component and virus. Standard proteins were located by staining a gel slice which was later autoradiographed to locate the virus polypeptides. The electrophoretic mobilities of the standard proteins were plotted against their molecular weights and values for the five radioactive bands determined by the method of Shapiro et al. (1967).* A typical plot of such data is shown in Fig. 4-11. The average molecular weights of the capsid polypeptides were calculated from four such determinations. In all gels examined in this way, both VP0 and VP1 migrated faster than pepsin, and so have molecular weights smaller than that of the latter, which is taken to be 33,600 daltons (Fruton, 1971). This finding contrasts with the generally accepted value of 35,000 daltons for VP1 (Maizel & Summers, 1968; Jacobson et al. 1970) and agrees with values of Wright (1973) determined using radioactively-labelled internal standards. The values of other poliovirus polypeptides showing similar electrophoretic mobilities were then determined relative to these values, and are recorded in Table 1 found in the Appendix.

(c) Discussion
In this section (4.6) an attempt has been made to obtain more accurate molecular weight values for the polypeptides of poliovirus. Experience with gel electrophoresis has shown that the sample load affects relative mobilities and so internal markers only have been used for measurements in this work. Errors may have arisen in previous determinations because of this, particularly for the measurement of the primary translation product of poliovirus RNA where the data relating its size to that of myosin have not been presented in the one gel (Summers et al. 1972) or have not been presented at all (Jacobson et al. 1970). Also, all samples have been treated identically prior to electrophoresis to ensure similar oxidation states and similar exposure to SDS for all polypeptides. In both previous determinations (Maizel & Summers, 1968; Jacobson et al. 1970) standards were not reduced with respect to disulphide bonds and so such protein - SDS complexes in these conditions were unlikely to migrate as unique functions of their molecular weights (Reynolds & Tanford, 1970). Other aspects of the method which are still subject to variation are the choice of suitable standards (and their molecular weight values) and the fact that in some variations of the SDS-gel procedure, some polypeptides, at least, do not migrate according to their size alone (see Chapter 5). For reasons to be discussed later, it is believed that determinations of molecular weights following electrophoresis in the presence of urea as well as SDS, are closer to the real values.

The theoretical coding capacity of poliovirus RNA is about 2,500 amino acids or 270,000 daltons of protein
Cooper et al. 1971). The largest poliovirus polypeptide detected in these experiments is about 210,000 daltons and in no experiment is there evidence of any radioactivity in any higher molecular weight regions. It accordingly seems likely that this molecule is the primary translation product of poliovirus RNA. Consequently, only about 80% of the RNA appears to be translated, and approximately 1500 nucleotides remain untranslated. Ninety nucleotides of this unused coding potential can be explained in terms of tracts of poly-A known to be attached to the 3' end of poliovirus RNA (Yogo & Wimmer, 1972). The "untranslated" RNA may be located at either or both ends of the message. This portion could, in theory, code for protein with a maximum molecular weight of approximately 55,000 if there were more than one site for initiation of translation in poliovirus RNA, but this situation seems unlikely since TPCK can prevent the appearance of all polypeptides smaller than 80,000 daltons (Fig. 4-6). There is no evidence in any of these experiments for more than one primary translation product.

The evidence of Yogo & Wimmer (1973) suggests that the chain-length of RNA in the poliovirus replicative form may be even longer than the virion RNA itself. This additional RNA is probably involved in the RNA replication process, and untranslated virion RNA may have a similar function in the priming of transcription or translation.

4.7 Summary

Certain amino acid analogues were screened for effects on the cleavage mechanism of poliovirus proteins. DL-ß-phenylserine and L-canavanine sulphate were found to be very potent inhibitors of cleavage and had similar effects in this regard even though they were analogues of different
amino acids. The effectiveness of four other analogues in inhibiting cleavage is offered as the first evidence for their incorporation into eucaryotic proteins. Canavanine in very low concentrations was found to be able to prevent viral RNA synthesis when introduced soon after infection.

Three new inhibitors of proteolytic enzymes with different activities were shown to interfere differently with the cleavage mechanism.

More accurate measurements of the molecular weights of poliovirus polypeptides were made. The size of the primary translation product of poliovirus RNA produced by three different methods was found to be 210,000 daltons. A consequence of this finding is that approximately 20% of the poliovirus RNA appears to be untranslated.
CHAPTER FIVE

ANOMALOUS ELECTROPHORETIC BEHAVIOUR OF SOME POLIOVIRUS
POLYPEPTIDES IN VARYING CONDITIONS OF SDS-GEL ELECTROPHORESIS

5.1 Introduction
5.2 The Effects of Urea on the Migration of Poliovirus Polypeptides
5.3 The Effects of Two other Modifications on Electrophoretic Mobilities
5.4 Evidence that the VP3 Region is Comprised of One Principal Amino Acid Sequence
5.5 Discussion
5.6 Summary
<table>
<thead>
<tr>
<th>Gel Component</th>
<th>Original Method of Summers et al. (1965)</th>
<th>Modified Method of Maizel (1971)</th>
<th>Standard Method used in this Thesis</th>
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<td>-</td>
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Table 4. Comparison of acrylamide gel compositions used by Summers et al. (1965), in common use (Maizel, 1971) and used in this thesis (based on Russell & Skehel, 1972).
5.1 Introduction

The electrophoresis of samples in SDS-containing polyacrylamide gels has been a popular method used for the analysis of proteins from a wide variety of biological sources. By this method, difficult samples can be easily dissolved and polypeptides separated on the basis of their size. Success with the technique has led to increasing confidence and the introduction of improvements or modifications to the original method (Summers et al. 1965). The work described in this chapter shows that such modifications may lead to artifacts, or to conditions in which some polypeptides do not migrate as a function of their size alone.

5.2 The Effects of Urea on the Migration of Poliovirus Polypeptides

The polypeptides VP0 and VP1 are generally well separated on traditional SDS-acrylamide gels (Summers et al. 1965; Jacobson & Baltimore, 1968b). Although the autoradiographic technique used in this thesis generally provided improved band resolution, difficulty was often encountered in satisfactorily separating these two polypeptides. In contrast, using either purified virions or whole cell extracts as samples, resolution of the VP3 region of gels into more than one radioactive band could often be achieved (Fig. 3-3). These observations suggested that the conditions of gel electrophoresis used in the present work were producing altered relative mobilities in some polypeptides. The gel composition used was similar to that described by Russell & Skehel (1972) and is compared (Table 4) with that used originally by Summers et al. (1965), and the widely-used modification of Maizel (1971).
Fig. 5-1 Electrophoresis in 10% gels of two samples in varying concentrations of urea. Alternate samples were prepared from a culture of whole infected cells, and an artificial mixture of virus and empty capsids.
Preliminary results showed that urea was the only variable in the system to cause alterations in the relative mobilities of poliovirus polypeptides. To investigate this effect further, pairs of gels were cast with final urea concentrations of zero, 1 M, 2 M, 3 M, 4 M and 5 M. Two samples were used in this experiment, each being treated as usual with SDS, 2-mercaptoethanol and urea. One sample was a digest of whole infected cells labelled for 1 h with $^{35}$S-methionine and the other was an artificial mixture of purified virus and empty capsids labelled similarly. Aliquots of each sample were electrophoresed in parallel in the gels containing varying concentrations of urea (Fig. 5-1). The most outstanding feature of this autoradiogram was that as the urea concentration increased, the relative separation of VP0 and VP1 in both preparations decreased. This region was complicated in the gels of infected cell extracts as at least two other polypeptides showed similar electrophoretic mobilities. PP29 migrated slightly slower than VP1 and could only be resolved adequately in gels containing urea at concentrations greater than 3 M; its mobility in lower concentrations was uncertain due to increasing complexity of the pattern. In contrast, PP31 apparently co-migrated with VP1 in high urea concentrations but could be resolved from VP1 in its absence, migrating midway between VP0 and VP1. Unfortunately, the capsid polypeptides in the gel without urea showed different absolute mobilities to those in the whole cell preparation and so corresponding bands did not align exactly.

The apparent multiple banding of the polypeptide VP3 in both samples could be seen in gels containing more than
The effect of acid-treatment of urea solutions on electrophoretic mobilities. All gels (10%) contained the concentrations of acid-treated urea shown. The sample used in the left-hand set was disrupted in the presence of SDS, 2-mercaptoethanol and acid-treated urea, while that on the right, in the presence of SDS, 2-mercaptoethanol and stored urea.
of urea may have been caused by contaminating cyanates either in the sample or in the gel itself. The following experiments were designed to examine this possibility. Cyanates are acid-labile and so a portion of the same urea solution was treated at pH 2 for 1 h and then neutralised (Stark et al. 1960). This solution was used immediately to prepare two sets of gels with varying concentrations of urea. Two portions of a sample of protein precipitated from infected cytoplasm with trichloracetic acid and then washed with ethanol were used as gel samples. One was treated with SDS, 2-mercaptoethanol and acid-treated urea while the other was treated with similar disrupting fluid made from the stored urea solution. Aliquots of the two samples were electrophoresed in parallel (Fig. 5-2). The unusual behaviour of the polypeptides in the VP0 and VP1 region is repeated with both sample pretreatments. Minor differences continue to be seen in some of the higher molecular weight polypeptides but unfortunately, many of the low molecular weight species have been lost during protein precipitation.

The most significant result from this experiment is that the multiple banding of the VP3 region is absent. Experiments using either fresh urea solutions or urea solutions passed through a mixed bed ion exchange resin ("ELGASTAT") gave similar patterns over-all, allowing only one VP3 band to be seen. These results suggested that cyanates present in the gel may have been the cause of the multiple banding of this polypeptide. However, the other effects seen in the presence of urea were not removed by pretreatment and are concluded to be due to intrinsic properties of the urea. Fig 5-2 also confirmed that the
presence of either type of urea in the sample pretreatment as opposed to the gel itself, did not alter the final polypeptide pattern. This result was suspected from Fig. 5-1, where any effect caused by the pretreatment could only be detected in urea-containing gels. If cyanate ions were present in the disrupting fluid used for pretreatment, 2-mercaptoethanol would quickly react with and remove them. This argument therefore eliminates the plausible explanation that cyanates from the urea were binding to reactive groups during heating and so causing protein modifications. Any significant alterations to proteins, whether they be caused by cyanates, urea or an unknown factor, must occur immediately after they enter the gel. This is shown by the generally well-defined features of the bands concerned, rather than a single, diffuse band.

To confirm that cyanates were not the cause of electrophoretic anomalies, further experiments were done to exclude this possibility. The concentration of cyanate ions in 10 M solutions of urea stored for more than 30 days at room temperature was found to be 12 mM by the method of Wrigley (1972). Similar solutions of urea either freshly prepared, acid-treated or purified by passage through an ion exchange resin contained cyanate concentrations less than 1 mM. Freshly prepared and acid-treated urea solutions were used to cast gels containing 5 M-urea. To exaggerate any effects cyanates may have had, more gels were cast containing, in addition, either potassium cyanate or ethanolamine (to react with cyanates) at final concentrations of 20, 50 and 100 mM. Aliquots of a sample of \(^{35}\)S-methionine-labelled poliovirus polypeptides were electrophoresed in parallel in
Electrophoresis in 10% gels containing added ethanolamine or potassium cyanate. The first three gels from the left of each set contained SDS alone, SDS + 5 M acid-treated-urea or SDS + 5 M freshly-prepared-urea respectively. The next three gels contained 5 M freshly-prepared-urea plus either ethanolamine or potassium cyanate at concentrations of 20, 50 or 100 mM respectively. The sample used was $^{35}$S-methionine-labelled poliovirus polypeptides from whole infected cells, treated with SDS and 2-mercapto-ethanol alone.
Addition of cyanates to urea-containing gels at concentrations up to 16 times that likely to be present as an impurity did not cause any alteration to the polypeptide pattern. Ethanolamine was added to gels containing concentrated urea to eliminate any free cyanates. None of the effects attributed to urea was abrogated by this addition, but two new electrophoretic changes (indicated by arrows) were observed in the presence of ethanolamine. The change in the higher molecular weight polypeptide caused it to migrate slightly faster while the change in the other apparently reduced its relative mobility. The nature of neither of these changes is understood, but the result demonstrates how the addition of a simple molecule can selectively alter the electrophoretic mobility of certain polypeptides. The results of both these experiments indicated that cyanates were not the cause of either the multiple banding of the VP3 polypeptide seen earlier or the comigration of polypeptides in the VP0 - VP1 region.

Electrophoresis even in the presence of purified urea led to alterations in the relative mobilities of some polypeptides (Fig. 5-2). Of the capsid polypeptides VP0 and VP1, at least one was behaving differently in the presence of urea and was not migrating as a function of its size alone. To clarify this point, $^{35}$S-methionine-labelled capsids were co-electrophoresed with four standard proteins in two gels, one of which contained 5 M-urea. The standard proteins were located by staining and the radioactive proteins by autoradiography of the stained, sliced gel. For each gel type, the mobilities of the standard proteins were plotted
The molecular weights of standard proteins plotted on a logarithmic scale against the distance migrated in gels with and without added urea. The mobilities of the capsid polypeptides are indicated and their average molecular weight values are shown in Table 5. TMV = tobacco mosaic virus protein.
against the logarithms of their respective molecular weights (Shapiro et al. 1967). In both gel types, the standards yielded relatively straight lines (Fig. 5-4). The average molecular weight values for the five structural polypeptides derived from four such plots are shown in Table 5.

The values obtained for VP1, VP3 and VP4 did not differ markedly when urea was included in the gels. However, the values for VP2 were consistently 1,000 to 2,000 daltons lower in the presence of urea, and values for VP0, 5,000 to 6,000 daltons lower, showing that the relative mobilities of both proteins were altered. Fig. 5-1 shows that this process varies progressively and does not occur at a critical urea concentration. The extent of the variation in molecular weight shown by VP0 between the two conditions is 14 - 17% which considerably exceeds the generous confidence level of 10% suggested by Dunker & Rueckert (1969) for molecular weight determinations done in SDS-acrylamide gels. Until more satisfactory methods are found for the molecular weight determinations of proteins, doubt will exist as to which is nearer the correct value. However, it is a reasonable expectation that a protein that is not fully denatured will not be fully accessible to interaction with SDS, which is the main determinant of mobility in this system. The view taken in this thesis is that the effect on VP0 of urea plus SDS allowed the denaturation of non-covalent bonds that were resistant to SDS alone, and that its electrophoretic mobility in the presence of both SDS and urea more closely reflected its molecular weight relative to the standard proteins.
### Table 5

<table>
<thead>
<tr>
<th>Poliovirus Polypeptide</th>
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<tr>
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<td>VP3</td>
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<tr>
<td>VP4</td>
<td>&lt; 10,000</td>
</tr>
</tbody>
</table>

Table 5. Average molecular weight values for poliovirus polypeptides determined in the presence or absence of 5 M-urea. Values are the averages from four such determinations from plots similar to those in Fig. 5-4.
Fig. 5-5 Electrophoresis in 10% gels of samples in varying concentrations of formamide. Sample used for the gels on the extreme left and right was purified virus. Sample used for other gels was $^{35}$S-methionine-labelled poliovirus polypeptides from whole infected cells. Both samples were treated with SDS and 2-mercaptoethanol alone before electrophoresis.
5.3 The Effects of Two other Modifications on Electrophoretic Mobilities

Urea is apparently able to complement the denaturing properties of SDS with regard to some polypeptides. Certain alternative protein solvents (dimethyl formamide and dimethyl sulphoxide) were found to interfere with gel polymerisation and were of little use. However, formamide was a feasible substitute up to 10 M concentrations even though gel polymerisation was very slow above 6 M. Aliquots of a sample containing $^{35}$S-methionine-labelled poliovirus polypeptides were electrophoresed in gels containing from zero to 10 M-formamide (Fig. 5-5). Behaviour similar to that seen for urea was apparent. The polypeptides in the region VP0 to VP1 were well separated in the presence of SDS alone, but comigrated in the presence of 6 M-formamide or above. Similar, less well defined changes in relative mobility were seen in both higher and lower molecular weight polypeptides, and also in the separation of the three major polypeptides in the samples of purified virus. It is concluded that in the presence of SDS, formamide, like urea, can alter the physical state of some polypeptides as shown by altered electrophoretic mobilities.

Urea (and formamide) may interact with SDS forming a complex when in the presence of water and protein. Such a complex may be disturbed by alteration of the ratio of urea to detergent. To test this possibility, two sets of gels were cast with concentrations of SDS of 0.1, 0.2 and 0.5%. One set of gels contained freshly prepared 5 M-urea. Aliquots of a sample of $^{35}$S-methionine-labelled infected
Electrophoresis in parallel in 10% gels containing varying concentrations of SDS. Gels on the left contained SDS alone while those on the right contained 5 M-urea as well. Concentrations of SDS in the gels are indicated. Concentration of SDS in the tank electrolyte was 0.5%.
cells were electrophoresed in the six different gels using 0.5% SDS in the tank electrolyte (Fig. 5-6).

At constant urea concentration, variation of SDS concentration in the gels had no effect on the relative separations. In the presence of 5 M-urea, the patterns were similar to those seen previously (Fig. 5-2). However, in the absence of urea, the patterns differed in that six bands could be seen in the region VP0 to VP2 whereas only four had been resolved previously (Fig. 5-1 and Fig. 5-2). Exactly which of these six bands corresponded to the previously described polypeptides is not known as direct comparisons are not possible using different electrolyte compositions. The additional bands may represent polypeptides obscured in previous separations, or may be artifacts.

This experiment failed to show that interactions between urea and detergent caused the altered electrophoretic mobility of SDS-protein complexes. It was concluded that variation of the SDS concentration in the tank electrolyte could lead to different separations of some polypeptides, but that this effect was masked in the presence of concentrated urea.

5.4 Evidence that the VP3 Region is Comprised of One Principal Amino Acid Sequence

The VP3 region of whole infected cell and of purified virus samples was often resolved into more than one band by gel electrophoresis in the presence of urea (see Chapter 3 and Fig. 5-1). This effect was never seen in the absence of urea and so the true number of polypeptides in this region was in doubt. If two or more unrelated polypeptides were present in this region displaying very
Fig. 5-7 Re-electrophoresis in 12% gels of radioactivity eluted from the VP3 regions of acrylamide gel separations of purified virus and infected cells. Gels on the right contained 5 M-stored urea as well as SDS. The samples used in both sets of gels were (a) whole infected cells, (b) purified virus, (c) VP3 eluted from gels of purified virus, and (d) VP3 eluted from gels of whole infected cells.
similar molecular weights, it seemed likely that such a situation would be revealed by an analysis of the component tryptic peptides. However, such an analysis would be unlikely to detect minor differences between two polypeptides which differed only in the terminal regions of their amino acid sequences. Such similar polypeptides could conceivably have been the result of ambiguous cleavages.

Samples from whole infected cells labelled with $^{35}\text{S}$-methionine were electrophoresed in twelve gels in the absence of urea. Samples of $^{35}\text{S}$-methionine-labelled virus purified on a sucrose gradient were electrophoresed similarly. The preparative procedure described in Chapter 2 for the detection, elution and precipitation of specific polypeptides from gels was followed for the preparation of polypeptides from the VP3 region of both sets of gels. One portion of each sample was used for the preparation of a tryptic digest (see Chapter 2) while another was treated with SDS, 2-mercaptoethanol and stored urea prior to re-electrophoresis. Both of the eluted preparations were analysed in gels either in the absence of urea or in the presence of 5 M-urea stored for an indefinite period (Fig. 5-7). Both sets of gels were pre-electrophoresed for 2 h at 8 mA per gel prior to the application of the sample. No difference could be seen between the two eluted samples. Both re-electrophoresed similarly and showed no sign of having been degraded during elution. In the presence of urea, both eluted samples showed a broad distribution of radioactivity, while the control samples showed multiple banding of the VP3 region. Hence, pre-electrophoresis of the gels did not abrogate this phenomenon. This experiment showed that the two eluted
Fig. 5-8 Comparison by electrophoresis on cellulose acetate of the $^{35}$S-methionine-labelled tryptic peptides derived from material eluted from the VP3 regions of acrylamide gel separations of purified virus or whole infected cells. (i) Comparison of the two digests separated at pH 6.5 (ii) Comparison of the two digests separated at pH 3.6. Details of the method are given in Chapter 2, and the position of the origin in each case is indicated.
Fig. 5-9 Comparison of $^{35}$S-methionine-labelled tryptic peptides derived from (i) the VP3 polypeptide eluted from acrylamide gels of purified virus, or (ii) the VP3 polypeptide region eluted from acrylamide gels of whole infected cells. Electrophoresis (horizontally) and chromatography (vertically) were done as described in Chapter 2. The position of the origin (O) and the final location of the phenol red marker (PR) included with the sample are indicated.
samples were likely to be identical but did not exclude the possibility that more than one polypeptide was present in each sample.

Tryptic digests of the two eluted samples were prepared (Chapter 2) and analysed in one dimension by electrophoresis on cellulose acetate strips at pH 6.5 and pH 3.6. The $^{35}$S-methionine-containing tryptic peptides were located by autoradiography (Fig. 5-8). No differences were resolved between the two samples at either pH.

Improved separation of the radioactive peptides was provided by two-dimensional paper electrophoresis and chromatography (Fig. 5-9). Both maps are very similar with apparent correspondence of the major radioactive spots. By cutting out the radioactive spots and quantitating each by scintillation counting, it was seen that each of the minor spots seen in the map from the VP3 region sample contained less than 20% of the average radioactivity of each of the peptides numbered 1, 2, 3, 4 and 6. This result suggested that the minor spots did not represent complete tryptic peptides, but were either products of alternate cleavages, or spurious degradation products. The peptide number 5 contained twice the radioactivity of the other major peptides, indicating that it either contained two methionine residues, or that two peptides were not resolved by this analysis. On this basis, the VP3 polypeptide probably contains seven methionine residues, or one for every 3,500 daltons of protein. Thus VP3 has a slightly richer methionine content than the average for the poliovirus structural proteins which have one methionine residue for every 4,000 daltons of protein, based on a methionine content of 2.7 mol per cent (Munyon & Salzman,
1962; Cooper & Bennett, 1973). This observation suggests that only one polypeptide comprises the VP3 region of gels rather than two (or more) as a figure of one methionine for every 2,000 daltons (or less) would be expected for the latter case.

This analysis does not exclude the possibility that two (or more) polypeptides having a major portion of their sequences in common comprise the VP3 region in gels. Such polypeptides may be the result of ambiguous cleavages during synthesis (Cooper et al. 1970) in which minor differences not involving methionine residues occur in end regions of the polypeptide. If this is the case for VP3, then differences in such polypeptides are demonstrable in only certain electrophoretic conditions.

5.5 Discussion

Urea has been used in various concentrations as an additive to SDS-gels for the examination of the polypeptides of several animal virus systems (Zweerink & Joklik, 1970; Russell & Skehel, 1972; Skehel, 1972; Bachrach & Hess, 1973). Neither the potential usefulness of urea as a means of obtaining alternative separations of polypeptides, nor the fact that molecular weight values sometimes vary even in low concentrations of urea has been reported previously. Consequently, a preliminary investigation of the phenomenon was considered appropriate in this thesis.

The addition of urea to SDS-gels was shown to alter the relative mobilities of some poliovirus polypeptides. The most pronounced effect was demonstrated in polypeptides with molecular weights around 30,000 daltons, possibly because the 10% gel concentrations chosen showed optimal
resolution for such polypeptides. The choice of more dilute or more concentrated acrylamide gels would have been able to show more clearly such anomalies in higher or lower molecular weight regions respectively. PP90 and PP8 are examples of polypeptides that show altered electrophoretic mobilities in the presence of urea (see Figs. 4-10 and 5-1).

The cause of the altered migration for some polypeptides is uncertain. VP0 exhibits sharp banding characteristics under all conditions indicating that the change in relative mobility is a gradual one. This could be explained by postulating that the increasing urea concentrations caused a gradual unfolding or relaxation of the polypeptide conformation allowing both a change in molecular radius and an increase in the amount of SDS bound to the polypeptide. The latter effect would give a larger molecular charge, leading to faster migration. Such an hypothesis is reasonable as the protein denaturing properties of urea and SDS differ; urea relaxes preferentially regions that are strongly hydrogen-bonded while SDS binds preferentially to hydrophobic regions. Support for this idea comes from the observation that all poliovirus polypeptides that show altered relative mobilities in the presence of urea or formamide migrate faster in such conditions. These experimental conditions have the effect of lowering the apparent molecular weights of these polypeptides.

The work of Reynolds & Tanford (1970) showed that SDS-polypeptide complexes in reducing conditions have a characteristic rod-shaped structure such that the molecular length is proportional to the molecular weight. The accompanying report of Fish, Reynolds & Tanford (1970) showed that
in non-reducing conditions where polypeptides bind lower amounts of SDS (Pitt-Rivers & Impiombato, 1968), the Stokes radius (or effective molecular length) is smaller. In the present work, some poliovirus polypeptide-SDS complexes may resemble non-reduced SDS-protein complexes in that the fully relaxed structure is not attained. The addition of urea or formamide to such complexes may allow further denaturation and increased binding of SDS. This latter effect could explain the increased relative mobility of these polypeptides.

A corollary of the hypothesis is that molecular weights of such polypeptides estimated by the SDS-gel method are likely to be closer to the true value when determined in the presence of concentrated urea. For this reason, all molecular weight measurements quoted in this thesis were determined in such conditions. The values are not claimed to be absolute; there is some indication that certain polypeptides may migrate even faster in the presence of higher concentrations of urea. However, the values quoted are claimed to represent the true molecular weight values more closely than those determined in other conditions (Maizel & Summers, 1968; Jacobson et al. 1970). The type of electrophoretic variations shown in this chapter could partially explain why such large discrepancies in the molecular weights of poliovirus polypeptides have been observed previously.

While the exact cause of different electrophoretic mobilities is not known, the primary amino acid sequence of the proteins is not necessarily the prime influence. VP0 is the immediate precursor of VP2 and VP4 (Jacobson et al. 1970) and so has sequences common to both the smaller proteins, yet neither of the latter shows the gross
variation in electrophoretic mobility in the presence of urea as does VP0. Thus the cause of the difference must originate in the structure of VP0, which before cleavage appears more resistant to denaturation than its cleavage products VP2 and VP4. A possible corollary of this observation is that the cleavage producing VP2 and VP4 causes a necessary partial denaturation of VP0 that allows association of the RNA with the capsid proteins during virus maturation.

Urea solutions can cause at least two different types of effect on polypeptide migration, one of which is acid-labile, can be removed by ion exchange resins and is not present in fresh urea solutions. These latter properties suggest that contaminating cyanates may be the cause of this effect seen as the occasional multiple banding of VP3. However, several pieces of data discount this possibility. Firstly, if cyanates were reacting with some functional groups on VP3 causing small increases in molecular weight, the result would be a diffuse distribution of radioactivity trailing behind a leading band. Instead, the effect usually seen (Fig. 3-5 and Fig. 5-1) consists of one or more discrete bands migrating slightly slower than VP3. Secondly, if cyanates are reacting with VP3, they should react similarly with all other polypeptides - a fact that is not shown experimentally. Thirdly, pre-electrophoresis of gels containing urea should remove any charged cyanates present. Fig. 5-7 shows gels that had been so treated yet the multiple banding of VP3 persisted. Fourthly, addition of excess cyanates to gels failed to induce the effect artificially (Fig. 5-3).

The multiple banding effect of VP3 was not
produced by all urea solutions available. Attempts to induce deliberately the artifact in a particular batch by storage at 37°C, or for long periods, failed. As the analysis of tryptic peptides by three different methods suggested only one polypeptide was present, the most likely explanation for the multiple banding of VP3 seen in certain conditions is that it is a variable artifact of unknown cause which reflects some peculiarity in the nature of this polypeptide.

A previous report has claimed to have resolved the poliovirus structural proteins VP2 and VP3 into a total of five bands by gel electrophoresis (Vanden Berghe & Boeyé, 1972). Their methods involved the dialysis of samples for 24 h at 37°C against a solution containing 5 M-urea, 0.1% SDS and 0.1 M-phosphate buffer prior to electrophoresis. They reported their separations to be variable, and so their result had features in common with some of the present results. It now seems likely that the use of concentrated urea solutions during sample preparation (even though urea was not included in the gels) may have been the cause of the extra bands observed. Although Vanden Berghe & Boeyé (1972) claimed to have resolved VP2 into three components, the present work suggests that all bands arising as the result of artifact are derived from VP3 (see Fig. 5-1). Extensive investigations of these polypeptides in this thesis supports the original claim (Maizel, 1963) that only four polypeptide species are present in the poliovirion.

Use of concentrated urea in SDS-gels has several advantages. Firstly, it can allow alternative separation patterns for a complex mixture of proteins, and
lead to the detection of previously obscured polypeptides (eg. PP29 described in Chapter 3 was detected in this way). Secondly, it forms a more effective denaturant for some proteins. Thirdly, it has two technical advantages in that it allows the application of larger sample loads to gels (Zweerink & Joklik, 1970), and it makes easier the task of overlaying gels. The use of concentrated urea in gels can lead to presumed artifacts which can be easily overcome by using fresh urea solutions. This work shows it to be a useful modification to the SDS-gel system and offers an alternative check when the homogeneity of a polypeptide is in doubt.

Since this work was completed, a report has appeared (Bachrach & Hess, 1973) which demonstrates the separation of the structural polypeptide of San Miguel sea lion virus from that of another calicivirus, vesicular exanthema of swine. Such separations are achieved in SDS gels in the presence of 8 M-urea, but not in its absence. Also, the two envelope polypeptides of RossRiver virus can be separated in urea-SDS gels but not in SDS gels alone (R.S. Raghow, personal communication). Thus all these examples in which urea has caused an alteration in polypeptide migration have been of structural proteins of viruses which, in general, are more difficult to denature than globular proteins. It remains to be seen if such proteins have unique properties with respect to SDS or whether this phenomenon is more widespread.

5.6 Summary

Several variations in the SDS-gel electrophoresis system produced different relative separations of
certain poliovirus polypeptides, the most important of which was the addition of urea. One of the effects caused by urea was shown to be an artifact which could be eliminated. Another, more beneficial, effect of urea was to increase the relative mobilities of some polypeptides leading to a new separation pattern. It was postulated that urea, combined with SDS, was able to achieve a more complete denaturation of some polypeptides than SDS alone. It followed that molecular weights determined by gel electrophoresis in these conditions were likely to be closer to the true values.
CHAPTER SIX

INTER-RELATIONSHIPS AMONG SEVERAL LARGE POLIOVIRUS
POLYPEPTIDES DEMONSTRATED BY PEPTIDE MAPPING

6.1 Introduction

6.2 Comparison of Tryptic Peptide Maps of Several Poliovirus Polypeptides
   (a) The relative complexity of maps derived from PP110 and PP90
   (b) Comparison of maps derived from PP168 (TPCK) and PP168 (TLCK)
   (c) Comparison of maps derived from PP168 (TPCK) and PP155 (TPCK)
   (d) Comparison of maps derived from PP155 and PP79

6.3 Discussion

6.4 Summary
6.1 Introduction

The order of cleavage of the poliovirus translation product and its immediate cleavage derivatives is virtually unknown. Since almost all cleavages occur within 5 min of labelling (Chapter 3), kinetic studies of the disappearance and appearance of each polypeptide yield only limited information. Analysis of tryptic peptides of individual polypeptides gives more definite information about the cleavage pathway, and has been used to show relationships between pairs of polypeptides eluted from acrylamide gels (Jacobson et al. 1970; Summers et al. 1971). Such polypeptide mixtures were separated in one dimension only, which generally resulted in inconclusive resolutions. However, major differences between PP110 and PP79 could be demonstrated, as could apparent similarities between PP110 and capsid proteins. The latter evidence was used to confirm suspicions that PP110 was the precursor of the structural capsid proteins.

This chapter describes separations of $^{35}$S-methionine-labelled tryptic peptides by a two-dimensional analysis and represents a preliminary attempt to extend the knowledge of the cleavage pathway and to produce more conclusive data about the precursor-product relationships. Such two-dimensional maps are characteristic for each protein and conclusions regarding similarities and differences can be made with greater confidence. It was expected that determination of the initial cleavages would be essential to allow the elucidation of later cleavages. The results below demonstrate certain inter-relationships among the larger polypeptides accumulating in poliovirus-infected cells incubated in the presence of the protease inhibitors, TPCK and TLCK.
Comparison of $^{35}$S-methionine-labelled tryptic peptide maps of polypeptides PP155 (TPCK) and PP110 (TLCK). The samples were applied as spots at the origins marked as 0. Electrophoresis (horizontally) and chromatography (vertically) were done as described in Chapter 2. The final position of a phenol red marker applied with the sample is shown in the top left-hand corner of each map. Autoradiography was for 20 days.
6.2 Comparison of Tryptic Peptide Maps of Several Poliovirus Polypeptides

(a) The relative complexity of maps derived from PP110 and PP90

Results presented in Chapter 4 showed that as well as the very large polypeptide PP210, two other prominent polypeptides, PP168 and PP155, accumulated in the presence of the enzyme inhibitor TPCK. Also, the inhibitor TLCK caused the accumulation of a polypeptide which had the same electrophoretic mobility as PP168. Both of these inhibitors also reduce the rate of cleavage of PP110 and PP90 allowing their appearance in greater than usual proportions, while allowing PP79 to accumulate in normal amounts. Although both of these compounds have alkylating activities other than those inhibiting certain proteolytic enzymes (Shaw, 1967), it was assumed that they would not have reacted with the poliovirus proteins sufficiently to alter their peptide maps.

Two 10 ml cultures of poliovirus-infected cells were incubated for 3.5 h prior to the addition of either TPCK (0.12 mM) or TLCK (0.32 mM). Five minutes later, $^{35}$S-methionine (10 μCi/ml) was added to each culture and incubation continued for 1 h. SDS digests of the whole cells of each culture were electrophoresed in twelve gels to give polypeptide separations similar to those seen in Fig. 4-8. The required radioactive bands were then eluted and digested with trypsin as described in Chapter 2. Tryptic peptides were separated firstly by paper electrophoresis and then by chromatography (Chapter 2) prior to autoradiography.

Methionine-containing peptide maps of polypeptide PP155 (TPCK) and polypeptides PP110, PP90 and PP79 (TLCK)
Fig. 6-2 Comparison of the $^{35}$S-methionine-labelled tryptic peptide maps of polypeptides PP90 and PP79. Experimental details are as for Fig. 6-1.
are shown in Figs. 6-1 and 6-2. Considerable variation in the intensities of the spots exists. This observation is explained, in part, by the presence of occasional peptides containing two (or more) methionine residues, or the failure of the method to resolve two methionine-containing peptides. Confirmation of such possibilities was provided by quantitating the radioactivity in each of the spots from the maps by scintillation counting. For example, such results for the map of polypeptide PP110 showed that the two most intense spots (indicated by arrows) contained approximately twice the radioactivity found in other spots of average intensity. Also, many of the very faint spots seen were found to have variable amounts of radioactivity, considerably less than that found in typical spots of average intensity. This result suggested that such faint spots do not represent authentic tryptic peptides of the particular polypeptide but are possibly the result of incomplete or non-specific digestion by the trypsin.

Because doubt exists in most maps as to which spots represent authentic tryptic peptides, relatively little importance is given to the absolute number of peptides found for each polypeptide. However, such maps are useful for comparative purposes, as preliminary trials indicated that this mapping procedure was reproducible for preparations of the same polypeptide digested and analysed on different occasions.

Figs. 6-1 and 6-2 show that the maps derived from polypeptides PP110 and PP90 are more complex and contain more radioactive spots than PP155, even though the latter is considerably larger. Conversely, the map of the PP79 digest
is much simpler than that of PP90 even though the former polypeptide is only slightly smaller. These observations raised suspicions as to whether the maps from PP110 and PP90 are derived from single polypeptides. Support for this possibility is found in Fig. 6-1 where, after making allowance for irregular chromatography at the left-hand side of the PP110 map, every radioactive peptide seen in the digest of PP155 appears to have a corresponding counterpart in the digest of PP110. This result seems best explained by postulating the presence of more than one polypeptide species in the sample eluted from the PP110 region of the preparative gels.

An alternative explanation for the comparatively complex maps of PP110 and PP90 would be that both of these polypeptides have a relatively rich methionine content. This possibility appears unlikely at least for the former polypeptide, which is the precursor of the capsid proteins, and accordingly, must have a major portion of its amino acid sequence common to these proteins. The methionine content of capsid proteins is 2.7 mol per cent (Munyon & Salzman, 1962; Cooper & Bennett, 1973), which suggests that approximately 23 methionine residues are present in these proteins. Tryptic peptide analysis of the capsid proteins (Cooper & Bennett, 1973) revealed a minimum of 25 methionine-containing peptides. Both of these figures are considerably less than the minimum of 40 methionine residues which can be deduced from the number of prominent radioactive spots in the map from PP110. To account for this discrepancy, either each radioactive spot does not represent a unique methionine residue or that more than one polypeptide comprises the PP110
Fig. 6-3  Comparison of the $^{35}$S-methionine-labelled tryptic peptide maps of polypeptides PP168 (TLCK) and PP168 (TPCK). Experimental details are as for Fig. 6-1. Obvious differences between the two maps are indicated by arrows.
region in gels. When compared with the relatively simple maps found for PP79 and PP155, the latter possibility appears more likely.

(b) Comparison of maps derived from PP168 (TPCK) and PP168 (TLCK)

Methionine-containing peptide maps (similar to those described in the previous section) derived from the polypeptide PP168 synthesized in the presence of either TPCK or TLCK, are compared in Fig. 6-3. Four peptides (indicated by arrows) present in PP168 (TLCK) appear to have no obvious counterpart in PP168 (TPCK). Conversely, two peptides present in PP168 (TPCK) appear to be absent from PP168 (TLCK). These observations suggest differences in at least six methionine-containing peptides, implying that PP168 species formed in the presence of TPCK and TLCK, although the same size, are not identical. Since a majority of the radioactive spots appear to correspond in the two maps, it seems likely that they have a long amino acid sequence common to both, and each has a short, unique region at one end.

This evidence shows that TPCK and TLCK have different effects on the cleavage of poliovirus proteins and that in this case polypeptides having similar electrophoretic mobilities in acrylamide gels are not identical.

(c) Comparison of maps derived from PP168 (TPCK) and PP155 (TPCK)

Consideration of the sizes of the polypeptides PP168 and PP155 shows that each represents about three-quarters of the total poliovirus translation product of 210,000 daltons. Thus they must have a minimum of one-half of their sequences and a maximum of all but 13,000 daltons
Fig. 6-4 Comparison of the $^{35}$S-methionine-labelled tryptic peptide maps of polypeptides PP168 and PP155 synthesized in the presence of TPCK. Experimental details are as for Fig. 6-1.
of their sequences overlapping. The peptide maps of these polypeptides were compared to determine the extent of the overlap (Fig. 6-4).

Although PP168 would be expected to have slightly more methionine-containing peptides based on its larger size, this is not the observed case. The only definite difference between the two maps is the relative enrichment in PP155 (indicated by an arrow) of a group of peptides. It is concluded from this result that these two polypeptides overlap extensively, and are most likely not located at opposite ends of the poliovirus translation product.

(d) Comparison of the peptide maps derived from PP155 and PP79

Peptide maps derived from digestion of polypeptides PP155 (TPCK) and PP79 (TLCK) are compared in Fig. 6-5. All but one of the methionine-containing peptides (indicated by an arrow) seen in PP79 have a corresponding counterpart in PP155. Conversely, several methionine peptides that are seen in PP155 are absent from PP79. (These differences are not clearly seen in the photographs but are apparent on examination of the original autoradiographs.) This result suggests that the sequence represented by PP79 is almost wholly included within that represented by PP155. Because PP79 has been shown to be located towards the C-terminal end of the poliovirus translation product (Taber et al. 1971; Summers & Maizel, 1971), it follows that PP155 must be similarly located. The peptide maps shown in the previous section show only a minor difference between PP168 (TPCK) and PP155 (TPCK), and it therefore seems likely that PP168 (TPCK) is similarly located towards the C-terminal end of
Fig. 6-5 Comparison of the $^{35}$S-methionine-labelled tryptic peptide maps of polypeptides PP155 (TPCK) and PP79 (TLCK). Experimental details are as for Fig. 6-1.
the poliovirus translation product.

6.3 Discussion

The experiments described in this section were designed to provide evidence to allow the determination of the initial cleavages of the poliovirus translation product. Since the inhibitors of proteolytic enzymes, TPCK and TLCK, allowed the accumulation in quantity of large precursor polypeptides not normally seen in cells labelled for long periods, such polypeptides appeared to be suitable species with which to compare other smaller poliovirus polypeptides. This approach suffers in that it is not known whether these two inhibitors cause any chemical alteration to poliovirus polypeptides. Because of the vast excess of host proteins present in the infected cell and the very low concentrations of the inhibitors used, it is assumed that only a small proportion of newly-synthesized poliovirus polypeptides might be affected by any non-specific alkylating activity of the inhibitors.

Analysis of tryptic peptides is one of the most convenient methods for demonstrating sequence similarities or differences among proteins. The method is not absolute as the parameters of separation used invariably depend on the amino acid composition of the peptide rather than its sequence, but is useful in cases where sequence analysis is impossible. Previous analyses of individual poliovirus polypeptides have involved separations in only a single dimension (Jacobson et al. 1970; Summers et al. 1971). The two-dimensional technique used in the experiments described here is considered to provide better resolution of peptides, allowing more reliable conclusions. Although methionine-
labelled peptide maps are not fully representative of proteins as only some of the tryptic peptides are detected, it was considered that this disadvantage was outweighed by the convenience of the method and the relative simplicity of the maps, which assisted resolution.

When compared to the maps derived from other polypeptides of comparable size, the maps from PP110 and PP90 appeared to contain more radioactive spots than would have been expected. The possibility that PP110 is not a single polypeptide is supported by the facts firstly that its cleavage products (the capsid proteins) appear to contain considerably fewer methionine residues (Cooper & Bennett, 1973) than is indicated from the map of PP110, and secondly that a larger polypeptide (PP155, TPCK) appears to have all of its peptides represented in a map of PP110. The possible dual nature of PP110 has not been recognised previously, and so doubt is cast on the validity of previous conclusions based on tryptic digests of protein eluted from this region of gels (Jacobson et al. 1970; Summers et al. 1971). Two polypeptides of this approximate size could conceivably be produced if the primary translation product, PP210, is cleaved in the middle. However, the possibility cannot be excluded that the inhibitor TLCK may have altered the cleavage pattern allowing the accumulation of a second artificial polypeptide of the same size. Unfortunately, the poorer quality and the complexity of the map of PP90 do not allow any definite conclusions to be made in favour of more than one polypeptide being present in this band, but the latter possibility is suspected.

The polypeptide PP79 was shown to have extensive overlap with PP155 (TPCK) and because the latter is very
Fig. 6-6 Diagrammatic representation of the positioning of several large poliovirus polypeptides relative to the primary translation product.
similar to PP168 (TPCK), it follows that PP79 has sequences in common with PP168 also, although this combination of maps is not shown in any of the figures. As PP79 has been shown to be located near the C-terminal end of the poliovirus translation product (Taber et al. 1971; Summers & Maizel, 1971), PP168 and PP155 must be similarly located (Fig. 6-6). This conclusion contrasts with the speculations of Cole & Baltimore (1973) who suggested that these two polypeptides must be translated from opposite ends of the poliovirus genome. They claimed (without any direct data) that PP110 was included within PP168 and that PP79 was included within PP155, which meant that sequences common to both PP168 and PP155 would have a maximum overlap of about 80,000 daltons, using the molecular weight values determined in their laboratory. The experiments described here show that the overlap between these two polypeptides (Fig. 6-6) is far more extensive than they suggest (Cole & Baltimore, 1973).

It had been hoped that the results provided by peptide mapping would have provided leads to allow a tentative ordering of the initial steps in the cleavage pathway. Even with the knowledge that PP168 and PP155 were probably located at or near the C-terminal end of the translation product, no conclusive evidence could be deduced as to the ordering of smaller polypeptides within these molecules. Attempts have been made by various workers in the past to determine the cleavage pathway using the derived molecular weight values of polypeptides as a basis. Because of the additional number of polypeptides now known to be coded for by poliovirus (see Chapter 3), it seems that the in vivo situation is probably too complex to tackle in this way.
The results of preliminary peptide mapping described in this chapter suggested that the cleavage pathway may not be as simple as previous reports have indicated (Jacobson & Baltimore, 1968b; Cole & Baltimore, 1973). As well as clarifying some points, the results described also complicate efforts to assign positions in the genome to the various known polypeptides. If two polypeptides are represented by the gel band PP110, do they overlap at all? Are the large polypeptides accumulating in the presence of TPCK and TLCK identical to normal precursor molecules? Which of the fully cleaved polypeptides represent unique amino acid sequences? Such questions can only be answered following more extensive analyses.

6.4 Summary

Two-dimensional, methionine-labelled, tryptic peptide maps were prepared from several large poliovirus polypeptides. Evidence from these maps suggested that PP110 (and possibly PP90 also), synthesized in the presence of TLCK at least, was composed of more than one polypeptide. Dissimilarities in the peptide maps of PP168 (TPCK) and PP168 (TLCK) confirmed earlier conclusions that the two inhibitors, TPCK and TLCK, have different effects on the cleavage of poliovirus polypeptides. Similarities in the peptide maps of PP168 (TPCK), PP155 (TPCK) and PP79 (TLCK) showed that all three polypeptides had been translated from the same general region of the poliovirus genome, so that these two large precursors must lie at the same end of the poliovirus translation product as PP79.
CHAPTER SEVEN

ATTEMPTS TO LABEL IN VIVO THE INITIATION SITE OF TRANSLATION OF POLIOVIRUS RNA

7.1 Introduction
7.2 The Incorporation of N-formyl-\(^{35}\)S-methionine into Mammalian Cells
7.3 The Location on Transfer RNA of Radioactivity Derived from Exogenous N-formyl-\(^{35}\)S-methionine
7.4 Poliovirus Polypeptides Labelled in the Presence of N-formyl-\(^{35}\)S-methionine
7.5 Discussion
7.6 Summary
7.1 Introduction

Poliovirus RNA is believed to have only one initiation site for the translation of protein (Jacobson et al. 1970). If this site could be specifically labelled, it would be possible to determine which of the cleaved polypeptide(s) was located at the N-terminal end of the poliovirus polyprotein. Such information would add significantly to the knowledge of poliovirus genetics. Since this work was completed, use of the drug pactamycin has shown VP4 to be very close to the N-terminus of poliovirus polyprotein (Taber et al. 1971; Rekosh, 1972).

The mechanisms of initiation of protein synthesis in procaryotes and eucaryotes are similar and have been extensively reviewed (Lucas-Lenard & Lipmann, 1971; Haselkorn & Rothman-Denes, 1973). One unexpected similarity was that the initiating met-tRNA^{met F} from eucaryotes could be artificially formylated in vitro by a procaryotic enzyme, yet retain its unique ability to initiate translation in the eucaryotic cell-free system (Housman et al. 1970). In such cases, the formyl group introduced at the N-terminus of newly-synthesized polypeptides was retained due to the lack of a suitable formyl-transferase for its removal in the eucaryotic cell. If N-formylmethionine could be used as the initiating amino acid for protein synthesis in poliovirus-infected cells, this group would serve to confirm the number of sites for initiation of translation of the poliovirus RNA in vivo.

The ability of eucaryotes to use for initiation a formylated met-tRNA^{met F} found usually in procaryotes, demonstrated one aspect of the universality of such processes.
118.

Thus it was considered possible that eucaryotic cytoplasm may similarly recognise N-formylmethionine and bind it specifically to its own tRNA\textsuperscript{met} \textsuperscript{F}. The work described in this chapter examines some aspects of the utilization of exogenously-supplied N-formyl-\textsuperscript{35}S-methionine by mammalian cells, but shows that label from such a source does not enter protein in a formylated form \textit{in vivo}.

7.2 The Incorporation of N-formyl-\textsuperscript{35}S-methionine into Mammalian Cells

The chemical synthesis of N-formyl-\textsuperscript{35}S-methionine based on the method of Sheehan & Yang (1957) is described in Chapter 2. Care was taken to ensure almost complete formylation of \textsuperscript{35}S-methionine; any unformylated amino acid remaining should have been removed by the treatment with Dowex 50W resin (Chapter 2). Analysis of the labelled compound by paper chromatography generally revealed 5 - 10% of the radioactivity chromatographing identically with N-formylmethionine sulphotide, an oxidation product that could not be entirely avoided. The presence of low levels of free methionine could not be excluded due to poor chromatographic separation from the formylated derivative. Exhaustive treatment of preparations to remove free methionine never completely eliminated about 1% of radioactivity co-chromatographing with free methionine. It was concluded that this portion probably represented oxidation products of N-formylmethionine formed during chromatography (Chapter 2).

The incorporation of N-formyl-\textsuperscript{35}S-methionine into acid-insoluble material was tested in poliovirus-infected cells incubated for 3.5 h in medium lacking added methionine. To two portions of this culture was added the
Fig. 7-1 The incorporation of radioactivity from $^{35}$S-methionine and N-formyl-$^{35}$S-methionine into acid-insoluble material. Points represent the acid-soluble radioactivity in 20 μl of each of the cultures described in the text. (a) Incorporation of the two labelled precursors (b) the effect of pactamycin $\rightarrow$ and emetine $\rightarrow$ on the incorporation of $^{35}$S-methionine (c) the effect of pactamycin $\rightarrow$ and emetine $\rightarrow$ on the incorporation of N-formyl-$^{35}$S-methionine. Note the change of scale in panel (c).
drug emetine (100 μg/ml) and to another two, pactamycin (5 x 10^{-7} M). Two more cultures received no additions as controls. Immediately, one culture of each type was treated with {^{35}S}-methionine (1 μCi/ml) while the others were treated with the same radioactive concentration of N-formyl-{^{35}S}-methionine. The incorporation of radioactivity into acid-insoluble material in each culture was followed (Fig. 7-1). Panel (a) compares the utilization of both precursors in the control cultures. Similar experiments with several batches of labelled precursor confirmed that the incorporation of radioactivity from N-formyl-{^{35}S}-methionine was consistently 2 - 4% of that incorporated from {^{35}S}-methionine. This result appeared consistent with the premise that radioactivity incorporated from the former was involved in initiation only as the poliovirus translation product would be expected to contain about 50 internal methionine residues for every polypeptide chain initiated. Panels (b) and (c) show the effects of two inhibitors of protein synthesis. Emetine, a drug which stabilises ribosomes on messenger RNA (Pestka, 1971), inhibited incorporation from {^{35}S}-methionine immediately. In contrast, the concentration of pactamycin used allowed incorporation of methionine to continue for 5 - 10 min before total inhibition was achieved, because initiation of translation rather than ribosome tracking is inhibited at low pactamycin concentrations, an effect amplified by the relatively long messenger in poliovirus-infected cells (Taber et al. 1971). The two drugs did not show a differential effect with N-formyl-{^{35}S}-methionine, incorporation of which was prevented equally in both cases. This result could be interpreted to indicate that radioactivity incorporated
Fig. 7-2  The absorption from the culture medium of exogenous $^{35}$S-methionine $\circ$ and N-formyl-$^{35}$S-methionine $\bullet$. Panel (a) represents the incorporation of each into acid insoluble material in 20 µl samples, and panel (b) the disappearance of acid soluble radioactivity from aliquots of the cell-free culture medium.
from N-formyl-$^{35}$S-methionine was being used exclusively for initiation of translation.

To check if N-formylmethionine could enter cells as readily as methionine does, the ability of cells to absorb N-formylmethionine from the culture medium was examined. After incubation for 3 h in medium lacking added methionine, equal amounts of $^{35}$S-methionine and N-formyl-$^{35}$S-methionine of the same specific activity were added to parallel cultures. Portions of each culture were taken at intervals and assayed both for incorporation of radioactivity into acid-insoluble material, and for radioactivity remaining in the culture medium (Fig. 7-2). The rate of disappearance of N-formylmethionine from the medium was much slower than that of methionine, an effect mirrored by the greater incorporation rate of the latter. This result suggested that N-formylmethionine was less able to enter cells than methionine but the possibility remained that small amounts may do so and may be uniquely positioned at the initiation site for protein translation.

7.3 The Location on Transfer RNA of Radioactivity Derived from Exogenous N-formyl-$^{35}$S-methionine

The preceding results were sufficiently encouraging to warrant critical examination of the incorporation mechanisms. Any transfer of radioactivity from N-formyl-$^{35}$S-methionine to cellular protein should be mediated by transfer RNA. This fraction of labelled cells was therefore examined for the presence of the labelled precursor.

Two cultures each containing $6 \times 10^7$ cells were incubated in medium without added methionine and exposed to pactamycin ($5 \times 10^{-7}$ M) for 10 min prior to the addition
Fig. 7-3 Analysis of transfer RNA digested with ribonuclease from cells labelled with either $^{35}$S-methionine or N-formyl-$^{35}$S-methionine.
(a) Paper chromatogram of digest of $^{35}$S-methionine-labelled transfer RNA treated with dilute ammonia. A mixture of $^3$H-labelled methionine and methionine sulphoxide was counted differentially as an internal reference.
(b) Paper chromatogram of a ribonuclease digest of N-formyl-$^{35}$S-methionine-labelled transfer RNA treated with dilute ammonia. A mixture of $^3$H-labelled N-formylmethionine and N-formylmethionine sulphoxide was counted differentially as an internal reference.
(c) Paper electrophoresis of a ribonuclease digest of transfer RNA labelled with $^{35}$S-methionine. The origin is at fraction zero. Migration towards the anode (+) or cathode (-) is indicated.
(d) Paper electrophoresis of a ribonuclease digest of transfer RNA labelled with N-formyl-$^{35}$S-methionine.
of either $^{35}$S-methionine or N-formyl-$^{35}$S-methionine (2 $\mu$Ci/ml). After 30 min both cultures were washed and transfer RNA fractions extracted as described in Chapter 2. Both samples were treated with pancreatic ribonuclease (10 $\mu$g/ml) to leave amino acids coupled to the adenosine moiety known to be at the 3' terminus of all transfer RNA molecules. One portion of each sample was hydrolysed with dilute ammonia (pH 10.5 at 37°C for 1 h) to release the free amino acids, which were analysed by paper chromatography (Fig. 7-3a,b). Methionine and a small amount of its sulphoxide were recovered from the transfer RNA of cells labelled with $^{35}$S-methionine. However, radioactivity recovered from the transfer RNA of cells labelled with N-formyl-$^{35}$S-methionine chromatographed identically with methionine and its sulphoxide rather than with the formylated derivatives. This suggested that N-formyl-methionine either was not coupling directly to transfer RNA or was being deformylated immediately afterwards. To confirm this, the radioactive precursors coupled to adenosine in the remaining portion were analysed by paper electrophoresis at pH 3.5 (Fig. 7-3c,d). Both samples gave similar radioactivity profiles with the major peak having a similar electrophoretic mobility to that of methionyl-adenosine (Galper & Darnell, 1969). No peak of radioactivity corresponding to N-formylmethionyl-adenosine (fractions -6 to -8) was detected. A minor peak co-migrated with free methionine.

These results provided evidence that N-formyl-methionine was not being utilized directly in the synthesis of proteins in mammalian cells. The most likely explanations for the previous results were that trace amounts of free methionine existed in the N-formyl-$^{35}$S-methionine, or that
Electrophoresis in 7.5% acrylamide gels of poliovirus polypeptides labelled with either $^3$H-methionine or N-formyl-$^{35}$S-methionine. All conditions for electrophoresis were as described by Summers et al. (1965).
the latter was being deformylated prior to incorporation into protein.

7.4 Poliovirus Polypeptides Labelled in the Presence of N-formyl-\textsuperscript{35}S-methionine

As a final check on the nature of incorporated radioactivity from N-formyl-\textsuperscript{35}S-methionine, the protein products in poliovirus-infected cells were examined. Infected cells were incubated for 3.5 h and then either \textsuperscript{3}H-methionine of N-formyl-\textsuperscript{35}S-methionine was added to parallel cultures. One hour later, the cells were washed and cytoplasmic extracts prepared for acrylamide gel electrophoresis. Portions of both labelled preparations were co-electrophoresed on the same gel which was fractionated transversely and the two isotopes counted differentially (Fig. 7-4). All polypeptides appeared to be labelled to the same extent by both radioactive compounds. This result confirmed the suspicion that radioactivity being incorporated into protein from exogenous N-formyl-\textsuperscript{35}S-methionine was, in fact, entering as free methionine.

7.5 Discussion

In vitro experiments have shown that eucaryotic tRNA\textsuperscript{metF} when charged artificially with N-formylmethionine, can mimic the procaryotic type of initiation of protein synthesis by placing N-formylmethionine at the N-terminus of newly-synthesized polypeptide chains (Housman et al. 1970). After initiation, the formyl group remains in position in the eucaryotic system as no suitable formyl transferase exists in such extracts to remove it. Hence, introduction of such a group to eucaryotic proteins in vivo should allow the specific labelling of the N-termini of newly-synthesized polypeptides.
In view of the ambiguity already expressed by tRNA$^{\text{met F}}$, the direct coupling of N-formylmethionine to tRNA$^{\text{met F}}$ seemed a feasible possibility.

The level of incorporation of N-formyl-$^{35}$S-methionine into mammalian cells compared to that of $^{35}$S-methionine was such that the former may have been positioned uniquely at the N-termini of newly-synthesized polypeptides. That the incorporation of both was sensitive to classical inhibitors of protein synthesis (Fig. 7-1) showed that the normal mechanisms of protein synthesis were being used and that N-formylmethionine was not being added to completed cellular components.

Comparison of the effects of emetine with those of pactamycin showed a differential effect for methionine but not for N-formylmethionine (Fig. 7-1). However, results with the latter showed only a low level of incorporation of radioactivity above the background. Hence, conclusions about an initiating role for N-formylmethionine based on these results are doubtful.

The analysis of radioactivity bound to transfer RNA gave no indication that N-formylmethionine was accepted and held in that form. Only free methionine was detected coupled to transfer RNA, implying that the precursor had been deformylated either before or immediately after coupling. Chemical deformylation either in the culture medium or within the cell seemed unlikely, but enzymatic deformylation could not be discounted as mitochondria contain a formyl transferase required for the procaryotic-type of protein synthesis occurring in those organelles (Lucas-Lenard & Lipmann, 1971). If such deformylation did occur after coupling to transfer RNA,
then the coupling was not specific for the initiating transfer RNA as shown by the labelling of poliovirus polypeptides (Fig. 7-4). In this case, both $^3$H-methionine and N-formyl-$^{35}$S-methionine gave identically labelled polypeptides suggesting that radioactivity from the latter had been incorporated into internal methionine positions during protein synthesis.

The combined evidence from several experiments therefore suggests that mammalian cells are unable to incorporate N-formylmethionine directly into protein. Cells do not efficiently absorb this compound nor are they able to use it for initiation of protein synthesis. Although some radioactivity from exogenous N-formyl-$^{35}$S-methionine was incorporated into protein, it was in the form of free methionine. The possibility of contaminating $^{35}$S-methionine in the preparation was not discounted with certainty. The alternate possibility that $^{35}$S-methionine arose by deformylation of the parent compound, or one of its derivatives also existed.

7.6 Summary

Attempts were made to label specifically in vivo the site of initiation of translation of poliovirus RNA with chemically-formylated $^{35}$S-methionine. The results suggested that the small amount of incorporated radioactivity did not represent N-formylmethionine nor was it located exclusively at the N-termini of polypeptides. It was concluded that N-formylmethionine was unlikely to be used directly by mammalian cells as a precursor for protein synthesis.
BIBLIOGRAPHY


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### Table 1: The polypeptides synthesized by poliovirus. The apparent molecular weights determined following electrophoresis in gels containing 5 M urea as described in Chapter 4. References: (i) Summers et al. (1965), (ii) Jacobson & Baltimore (1968a), (iii) Jacobson & Baltimore (1968b), (iv) Jacobson et al. (1970), (v) Cole & Baltimore (1973), (vi) Summers et al. (1971). Polypeptides marked by an asterisk are those characterized in this thesis for the first time.

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