STUDIES ON THE MULTIPLICATION OF VIRUSES

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"Studies on the Multiplication of Viruses"

Myxoviruses and Paramyxoviruses

1. White, D. O., Day, H. M., Batchelder, E. J., Cheyne, I. M., and Wansbrough, A. J. (1965). Delay in the Multiplication of Influenza Virus. Virology 25, 289-302.

2. White, D. O., and Cheyne, I. M. (1966). Early Events in the Eclipse Phase of Influenza and Parainfluenza Virus Infection. Virology 29, 49-59.

3. White, D. O., and Cheyne, I. M. (1965). Stimulation of Sendai Virus Multiplication by Puromycin and Actinomycin D. Nature 208, 813-814.

4. Cheyne, I. M., and White, D. O. (1969). Growth of Para-myxoviruses in Anucleate Cells. Aust. J. exp. Biol. med. Sci. 47, 145-147.

5. Haslam, E. A., Cheyne, I. M., and White, D. O. (1969). The Structural Proteins of Newcastle Disease Virus. Virology 39, 118-129.

6. Taylor, J. M., Hampson, A. W., and White, D. O. (1969). The Polypeptides of Influenza Virus. I. Cytoplasmic Synthesis and Nuclear Accumulation. Virology 39, 419-425.

7. Haslam, E. A., Hampson, A. W., Egan, J. A., and White, D. O. (1970). The Polypeptides of Influenza Virus. II. Interpretation of Polyacrylamide Gel Electrophoresis Patterns. Virology 42, 555-565.

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9. Taylor, J. M., Hampson, A. W., Layton, J. E., and White, D. O. (1970). The Polypeptides of Influenza Virus. IV. An Analysis of Nuclear Accumulation. Virology 42, 744-752.

10. White, D. O., Taylor, J. M., Haslam, E. A., and Hampson, A. W. (1970). The Polypeptides of Influenza Virus and their Biosynthesis. In "The Biology of Large RNA Viruses" (R. D. Barry and B. W. J. Mahy, eds.), pp. 602-618, Academic Press, London.

Adenoviruses

[*]11. Maizel, J. V. Jr., White, D. O., and Scharff, M. D. (1968). The Polypeptides of Adenovirus. I. Evidence for Multiple Protein Components in the Virion and a Comparison of Types 2, 7A and 12. Virology 36, 115-125.

12. Maizel, J. V. Jr., White, D. O., and Scharff, M. D. (1968). The Polypeptides of Adenovirus. II. Soluble Proteins, Cores, Top Components and the Structure of the Virion. Virology 36, 126-136.

13. White, D. O., Scharff, M. D., and Maizel, J. V. Jr. (1969). The Polypeptides of Adenovirus. III. Synthesis in Infected Cells. Virology 38, 395-406.

[*]Paper 11 is not to be considered part of this submission for the degree of M. D., but is merely included as background information to make papers 12 and 13 more readily comprehensible. The author feels that his contribution to this particular paper was not substantial enough to justify inclusion in the collection.

INTRODUCTION

This collection of 12 papers published between 1965 and 1970, mainly in Virology, embodies the results of most of the author's research during those six years. Apart from a period of 10 months spent at the Albert Einstein College of Medicine in New York the work was conducted in the author's laboratory in the University of Melbourne.

A number of colleagues were direct collaborators at various stages. In acknowledging their invaluable contributions, it may be of interest to list them, indicating their role in the work of the laboratory. The extent of their involvement in the execution of the experiments is accurately reflected in the authorships. The work was planned and the papers written by DW, but several of the coauthors made important contributions at these stages also.

Miss Elaine J. Batchelder - B.Sc.Hons. student 1962-3.
(paper 1)
Mr. I.M. Cheyne - Ph.D. student 1963-68.
(papers 1,2,3,4,5)
Mrs. Hilary M. Day - M.Sc. student 1961-3.
(paper 1)
Miss Julie A. Egan - M.Sc. student 1969-70.
(paper 7)
Mr. A.W. Hampson - M.Sc. student 1968-70.
(papers 6,7,8,9,10)
Dr. Elizabeth A. Haslam - NATO Postdoctoral fellow 1967-70.
(papers 5,7,8,10)
Miss Judith E. Layton - B.Sc.Hons. student 1969.
(paper 9)
Dr. J.V. Maizel - Assistant Professor, Albert
(papers 11, 12, 13) Einstein College of Medicine 1966.
Miss Ieva Radiskevics - M.Sc. student 1969-70.
(paper 8)
Dr. M.D. Scharff - Assistant Professor, Albert
(papers 11, 12, 13) Einstein College of Medicine 1966.
Dr. J.M. Taylor - ARGC Postdoctoral fellow 1968-70.
(papers 6, 9, 10)
Miss Ann J. Wansbrough - Research assistant 1963-4.
(paper 1)

The central interest of the author's laboratory over the
last six years has been the mechanism of multiplication of
myxoviruses and paramyxoviruses. The early work attempted
to throw some light on the nature of the events of the eclipse
period that are blocked by inhibitors of transcription and trans-
lation. As more sophisticated techniques became progressively
available this led to direct experiments to demonstrate the
relevance of the nucleus in the multiplication of these viruses.
Successful identification of the structural and functional role of
each of the proteins of the virion provided the opportunity to
investigate their site of synthesis and subsequent migration
around the cell. The influenza-NDV programme was punctuated
in 1965-66 by a year at the Albert Einstein College of Medicine
where collaboration with an immunologist, M. D. Scharff, and
a chemist, J. V. Maizel, led to a detailed analysis of the fine
structure of the adenovirion. A brief outline of the major
findings of each paper is provided below in the context of the
state of knowledge at the time and the hindsight of subsequent
events.

Papers 1, 2, 3, 4

When this work was commenced in 1963 the new era of bio-
chemical virology was not yet in full flower. Techniques were
still somewhat primitive, and in Australia, where quarantine
restrictions prevented the importation of both fowl plague and
virulent Newcastle disease viruses, no satisfactory continuous
cultured cell line was available for the study of "myxoviruses".
The work of our own laboratory at the time therefore centred
around two other assay systems - "allantois-on-shell" (Fazekas

de St. Groth & White, 1958) and "haemadsorption to single cells
in suspension" (White et al, 1962). Some years earlier the
author had been interested (White 1959, 1960) in Cairns'
beautiful demonstration of "multiplicity-dependent delay" in
the multiplication of viruses (Cairns 1957, 1960). The work
reported in papers 1 and 2 stemmed from that interest but was
soon to lead us into a broader involvement with the multiplica-
tion of influenza virus in general. Our initial approach in 1963
involved the manipulation of inhibitors in quite intricate experi-
ments designed to determine the sequence of events in the cycle.

Multiplicity-dependent delay in the multiplication of
influenza virus was shown to occur after adsorption, penetration,
and the synthesis of at least one "early protein", but before the
last actinomycin D-sensitive event, the acquisition of resistance
to ultraviolet irradiation, the synthesis of viral structural
proteins, and the establishment of interference (papers 1, 2).

Viral multiplication in cells infected asynchronously by
single particles could be conveniently "synchronized" by pro-
longed fluorophenylalanine inhibition then reversal. FPA was
shown to block virus multiplication at two distinct points: high
concentrations interfered with "early protein" production,
whereas lower doses acted $1\frac{1}{2}$ -2 hours later on the synthesis
of viral structural proteins (paper 1).

Following the key observation (Barry et al, 1962) that the
multiplication of influenza virus, but not NDV, is inhibited by
actinomycin D, Barry (1964) had just reported that ultraviolet
irradiation of cells before infection also inhibited influenza
more markedly than NDV. Our own work about that time

(papers 1, 2) went on to show that both actinomycin and UV blocked an early event that follows the synthesis of at least one early protein but is complete by 2 hours post-infection. Our finding that the "capacity" (susceptibility to UV administered before infection) of the cells to support the growth of influenza virus is just as sensitive as the cell-virus complex itself during the first hour after infection, strongly suggested that some UV-sensitive (and actinomycin-sensitive) cell function, presumably transcription, is essential to the multiplication of influenza, but not Sendai or NDV. The conclusion that cellular RNA synthesis may be vital in the early stages of the replication of myxoviruses but not paramyxoviruses gained support from paper 4 in which the paramyxoviruses, parainfluenza type 1 and NDV, but not influenza, were grown in anucleate cells. Nevertheless, it is fair to say that the whole question of the role of the nucleus in the multiplication of myxoviruses is still unresolved today.

Less in dispute is the ability of the paramyxoviruses to multiply entirely independently of the nucleus and of cell-coded RNA. Barry's evidence to that effect is supported directly by the enucleation experiments (paper 4), the relative radioresistance of cells in Sendai virus infection (paper 2), and the finding that this virus actually grows more rapidly in actinomycin-treated than in normal cells (paper 3), all of which point to the genetic self-sufficiency of paramyxoviruses.

Papers 5, 6, 7, 8

At the end of 1966 it was decided to conduct all future experiments in cultured cells, despite the difficulties with influenza, so that advantage could be taken of modern techniques of cell fractionation to determine the site of synthesis and subsequent migration of viral nucleic acid and proteins. The hope was to develop a system that would not only throw light on the vexed question of the role of the nucleus in influenza viral multiplication but might also serve as a model for the study of the many other viruses that also involve the cell nucleus. For years it had been tacitly accepted that the proteins of such viruses, including influenza, are synthesised in the nucleus, which is where they are predominantly found.

Firstly, a protocol was devised (papers 5, 7) for the rigorous purification of pleomorphic and fragile enveloped viruses. Following concentration and gentle deposition onto a sucrose "cushion" the virus is banded first by rate zonal, then by equilibrium gradient centrifugation.

The technique of acrylamide gel electrophoresis in neutral SDS (Maizel 1966, 1969), which was being widely used to analyse the polypeptide composition of icosahedral viruses had to be adapted to cater for enveloped viruses, because some of their proteins turned out to be unusually resistant to dissociation by reducing agents. A systematic attack on this problem demonstrated these to be envelope glycoproteins held together by disulphide bonds that could be broken only under severe reducing conditions. Using these conditions it was shown

(paper 7) that influenza virus contains two major polypeptides and one major glycoprotein (composed of two distinct species of glycopeptide), plus a minor glycoprotein (also composed of two glycopeptides).

All six polypeptides of influenza were identified by dissociating purified radioactively labelled virions or infected cells with various detergents, then separating the resultant subunits by gradient centrifugation, and analysing their composition and function. Most of the procedures were worked out first on NDV (paper 5), and elaborated in considerably greater detail on influenza during the ensuing couple of years (papers 6, 7, 8). The two groups of viruses appear to be very similar in polypeptide composition but only the influenza strains A/Bel and B/Lee were analysed in real detail. The main findings were as follows.

The peplomer corresponding to the viral haemagglutinin was found to be a polymer (probably a tetramer) consisting of two molecules of a relatively cysteine-rich glycoprotein of MW about 77000, which could be broken down under rigorous reducing conditions to two glycopeptides of MW about 60,000 and 20,000 (papers 7, 8). Since the dissociation of the haemagglutinin glycoprotein into these two glycopeptides was reported, Mrs. Stanley, a graduate student in the author's laboratory, has confirmed that the breakdown is quantitative, yielding equal numbers of the two smaller molecules, that these two glycopeptides are distinct, and that the larger one contains twice as much carbohydrate (relative to protein) as the smaller (see Aust. Biochem. Soc. abstracts, Brisbane meeting, May 1971).

The neuraminidase is a relatively minor component of the virion, not conspicuous in most gel electrophorograms. However it did prove possible to purify the active enzyme from dissociated virions and to show that it too is a polymer of glycoprotein, which dissociates under rigorous reducing conditions to two glycopeptides (paper 8). Since these findings were reported, Miss Radiskevics has confirmed that two molecules of MW around 60,000 make up a polymer that can be found in purified preparations of neuraminidase, and that the active enzyme is in turn a polymer of this glycoprotein.

The nucleocapsid protein is a single polypeptide of MW 50,000 (paper 8).

A quite unexpected finding was the discovery of a major new viral constituent (VP3), a relatively arginine- and methionine-rich, low MW (21,000) polypeptide which is by far the most plentiful molecule in the virion (papers 6, 7, 8). On the basis of the finding that VP3 is readily released from the ribonucleoprotein "core" following treatment of the virus with deoxycholate it was postulated to be situated on the inside of the viral envelope, surrounding the RNP (paper 10). Support for this hypothesis has recently come from Mrs. Stanley's demonstration that, unlike the haemagglutinin and the neura- minidase which of course are situated on the outside of the virus, VP3 cannot be labelled with ^{125}I (enzymatically, using lactoperoxidase, MW 82,000) prior to dissociation of the particle (A. B. S. Abstracts, May 1971).

No host protein was found to be present in highly purified virus (papers 6, 8, 10). This fact, which has important impli- cations in respect of the mechanism of displacement of

pre-existing cellular proteins from the plasma membrane of
infected cells, has been demonstrated more directly and
unequivocally by Holland and Kiehn (1970). Their finding,,
together with our own (paper 7), that the electrophoretic
mobility of the glycoproteins, but not the other proteins,
varies for a given strain of influenza virus grown in different
species of host cell strongly suggests that all "host antigen"
in influenza virus is carbohydrate present in the envelope
glycoproteins, the protein of which is virus-coded but the
carbohydrate determined by the combination of transferases
present in the particular species of cell.

Papers 6, 9, 10

The finding (paper 6) that most of the protein synthesised
by infected cells consists of the structural proteins of the
virion that we could now so readily identify in gel electrophoro-
grams enabled us to turn our attention to the intracellular
synthesis and migration of these proteins. It was found (paper
10) that all three of the major peaks (the undissociated haemag-
glutinin glycoprotein VP1, the nucleocapsid polypeptide VP2,
and the new, low MW, arginine-rich polypeptide VP3) appeared
by 2 hours post-infection and were being synthesised at maximum
rate by 4 hours p.i., both in the permissive calf kidney system
and in the non-permissive HeLa cell system from which little
infectious virus is released. Throughout the cycle the three
are made in a constant ratio which does not, however, corres-
pond to that found in the virion itself; the ribonucleoprotein is
synthesised in excess. All the viral polypeptides are stable;
there is no evidence for a short-lived, high MW precursor.

Host protein synthesis begins to decline only after viral structural proteins begin to be made; by 4 hours about half of all protein being synthesised by the infected cell is viral.

Cell fractionation techniques were developed to ensure the preparation of nuclei completely free of cytoplasmic contamination (paper 6). Nuclei were released by Dounce homogenization, then cleaned with Nonidet P40 and spun through a dense solution of sucrose. Nucleoli were extracted by Penman's method. Plasma membranes were separated from rough endoplasmic reticulum by a modification of the methods of Hays and Barland involving flotation of membrane fragments through discrete layers of sucrose to equilibrium using isopycnic centrifugation (paper 10). These various procedures were used to fractionate cultured cells following short pulse-labelling with radioactive amino acids and "cold chases". The findings were as follows (papers 6, 9).

All the viral proteins were shown to be synthesised in the cytoplasm. The two relatively arginine-rich polypeptides, VP2 and VP3, subsequently accumulate in the nucleus. The migration was found to be uninfluenced by multiplicity of infection, or by the addition of cycloheximide or actinomycin D. The polypeptide of the nucleocapsid migrates relatively slowly, and is found mainly in the nucleoplasm, whereas the other arginine-rich polypeptide, which is of lower MW, migrates very rapidly and is found mainly in the nucleolus. This finding that two specific and readily identifiable polypeptides could be simply tracked in their movements around the cell provided us with the first really satisfactory experimental

system for studying the factors that influence the migration around mammalian cells of proteins in general.

It may be worth noting that Becht, who published a paper in 1969 purporting to show by autoradiography that an arginine-rich influenza protein(s) may be synthesised in the nucleus, has now found (personal communication) by using shorter pulses that these influenza proteins are in fact synthesised in the cytoplasm and migrate rapidly into the nucleus.

The glycoprotein of the haemagglutinin was found to move into plasma membranes (paper 10). Current work in the author's laboratory is directed towards determining the site, timing and sequence of attachment of various sugars to the polypeptides of VP1, and the mechanism of assembly of the haemagglutinin in plasma membranes.

Much of 1970 has been invested in trying to determine the site of synthesis of influenza viral RNA. Though consistent, results to date are, in the author's view, not yet sufficiently unequivocal to justify publication. Hybridization will therefore be used for definitive identification of positive and negative strands.

An attempt has been made in the foregoing pages to summarize most of the significant positive findings described in papers 1-10. This is not the place to propose purely hypo-thetical models for the multiplication of influenza. The author's speculations on such matters are spelt out at some length in the Discussion sections of all ten papers, the most recent synopsis being found at the end of papers 9 and 10.

Papers 11, 12, 13

During 1965-66 the author was fortunate to be able to spend a sabbatical year in Dr. H. Eagle's Department of Cell Biology at the Albert Einstein College of Medicine, New York. At the time Dr. J. V. Maizel had just developed a revolutionary method of acrylamide gel electrophoresis that enabled him to resolve dissociated adenovirions into an unexpectedly large number of distinct peaks thought to correspond to individual polypeptides (Maizel, 1966). In conjunction with Dr. M. D. Scharff and the author it was decided to attempt the complete molecular characterization of the virus. That we succeeded in doing this at a time when it had not been achieved for any other virus points up the advantages of close collaboration between people trained in completely different disciplines.

The first step was to establish, in the face of widespread skepticism, that each electrophoretic peak obtained by the Maizel method did indeed correspond to a unique polypeptide. This laborious task was undertaken entirely by Maizel (although the results form part of paper 11) by isotopic ratio analysis and comparison of a number of oncogenic and non-oncogenic serotypes. It was found that each was indeed a distinct polypeptide, provided that the dissociation of the virion by SDS was carried out at elevated temperature (e. g. boiling for one minute) to ensure the breakage of non-peptide bonds that link the polypeptides into capsomers.

The adenovirion was shown to have a much more complex structure than previously assumed. In paper 12, eight

different species of polypeptide were assigned a place in the particle. Three are present in an outer capsid, three in an inner core, and two others in association with hexons.

The hexon capsomer was shown to be composed of approximately three molecules of a single type of polypeptide of MW 120,000 which comprises about 50% of the total virion protein. Groups of such capsomers, released from the virion by 5M urea, are   associated with two minor polypeptides, each of MW 13,000.

The penton-base was found to be composed of a single type of polypeptide of MW 70,000, and the fibre of another polypeptide of MW 62,000.

The internal core released by treatment of the virion with 5M urea was demonstrated to contain the viral DNA in association with three arginine-rich polypeptides of MW 44,000, 24,000 and 24,000, comprising some 20% of the total virion protein. The same three polypeptides were relatively lacking in the "empty" capsids found as the "top components" of caesium chloride gradients of crude virus preparations.

It is pleasing to note that Laver (1970) has now confirmed the findings of paper 12 in virtually every detail, including the existence of nine polypeptides of the same MWs and the allocation of the same three polypeptides to a clearly demonstrable viral core.

In paper 13 attention was directed at the adenovirus-infected "spinner" HeLa cell. Hexons, pentons, fibres and cores, as well as virions and empty virions were isolated from infected cells by rate zonal centrifugation, DEAE-

cellulose chromatography and equilibrium gradient centrifu-
gation. Specific antisera were used to precipitate the various
types of radioactively-labelled capsomer, which were then
dissociated and analysed by gel electrophoresis.

It was found that all eight (or nine) structural polypeptides
of adenovirus type 2 could be identified in the infected HeLa
cell. All proved stable; none is a precursor of another. The
various polypeptides are synthesised at very different rates,
which do not reflect the ratio in which they are represented in
the virion itself. For example, the penton-base and fibre
polypeptides are made in considerable excess, whereas the
three polypeptides comprising the inner viral core are in
relatively short supply. At least 80% of the viral protein
found in infected cells is in the form of free hexons, pentons
and fibres.

Host protein synthesis was shown to proceed completely
normally until after viral capsid protein synthesis begins,
then to decline in parallel with the gradual rise in the rate of
viral protein synthesis. When viral DNA replication was
inhibited, no capsid proteins were made and no shutdown of
cellular protein synthesis occurred; even when limited produc-
tion of viral DNA and capsid protein was allowed, host protein
synthesis was little affected even many hours later. This led
to the hypothesis (first enunciated in another context in paper 3)
that the late inhibition of cell protein synthesis seen with many
viruses may be attributed to competition for ribosomes between
host and viral messenger RNA, rather than to a virus-coded
"cell shutdown" protein as seems to be the case in the "early"
shutdown by some viruses.

The ability to identify and measure particular proteins intracellularly seemed to provide the first real opportunity to study the stability of individual species of messenger RNA in mammalian cells. It was found that in the presence of actinomycin D the mRNA[s] for each of the structural poly-peptides of the adenovirion decayed with a mean half-life of 6 hours.

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Delay in the Multiplication of Influenza Virus[1]

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A chance delay, the duration of which varies inversely with multiplicity of infection, occurs early in the multiplication cycle of influenza virus. The delay takes place after the virus has penetrated beyond the reach of antibody, but before the first process inhibited by actinomycin D and before the synthesis of viral structural protein. Virus multiplication may be synchronized by the addition and subsequent removal of fluorophenylalanine.

Fluorophenylalanine blocks at two distinct points in the influenza virus multiplication cycle. High concentrations of the drug inhibit a process beginning very shortly after infection, whereas lower doses act 1½–2 hours later. A further 1½–2 hours elapse between the synthesis of a protein molecule and its emergence from the cell in the form of mature virus. Actinomycin D acts only early in the cycle and has no inhibitory effect on the synthesis of viral structural protein or any concurrent or subsequent process dependent thereon. The mode of action of actinomycin is discussed.

The progress of viral multiplication in suspension culture is readily followed using a technique of hemadsorption to single cells.

INTRODUCTION

When a population of cells is infected with influenza virus at an average multiplicity (m.o.i.) of less than 1, there is a considerable variation in the times at which different cells begin to produce new virus. A few yield virus after a certain fixed minimum interval, but most show an additional delay of anything up to several hours. Though the literature abounds with examples of such "asynchrony" in a variety of virus–cell systems, there have been relatively few deliberate investigations of its mechanism. The original formal description of the phenomenon was by Cairns (1957), who studied asynchrony in the multiplication of influenza virus in the

allantoic cavity of the egg. He was able to show that the extent of the delay in the case of any given virus–cell encounter depends solely on chance and may be reduced towards zero by progressively increasing the m.o.i. The duration of the delay is not predetermined in any heritable fashion by the virus or by the cell. In a sophisticated study of asynchrony in vaccinia virus infection Cairns (1960) demonstrated that several particles may set up in one cell distinct cytoplasmic "factories" where synthesis of viral nucleic acid and protein occur together. Cairns envisaged that, following a "delay," one particle (the "initiator") effects some critical step ("initiation") that triggers the simultaneous independent replication of all virus particles within that cell. Easterbrook (1961) demonstrated that the stage of vaccinia virus multiplication inhibited by azide separates a period of variable duration from one of constant duration. Now Joklik (1962, 1964a) has shown that rabbitpox virus is uncoated

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earlier under conditions of multiple infection or following preinfection with a related strain, and Cooper (in press) has demonstrated that about half the delay experienced by poliovirus occurs during penetration.

Throughout this paper the term "delay" will refer to only those random delays in the viral multiplication cycle attributable to chance and overcome at high m.o.i. An attempt is made to define the conditions under which delay occurs in the multiplication of influenza virus, to determine the effect of various inhibitors on delay, and to identify the stage or stages in the cycle at which delay occurs.

ABBREVIATIONS

The following abbreviations are used: $ID_{50} = 50\%$ infectious dose; AD = hemagglutinating dose; HA = hemagglutinin; m.o.i. = multiplicity of infection (expressed as infectious units per cell); p.i. = post infection; AOS = allantois-on-shell; SM = standard medium; CaMg-free PBS = calcium- and magnesium-free phosphate-buffered saline (Dulbecco and Vogt, 1954); RDE = receptor-destroying enzyme (neuraminidase); PA = DL-phenylalanine; FPA = DL-p-fluorphenylalanine; RNA = ribonucleic acid; DNA = deoxyribonucleic acid.

MATERIALS AND METHODS

Virus. BEL (Burnet and Bull, 1943) is an egg-adapted strain of influenza A. Stocks were grown in the allantoic cavity of 10- to 11-day-old embryonated eggs for 42 hours at 36° and stored at −65°. Before each experiment the virus was subjected to 1 minute's treatment in an M.S.E. 500-watt ultrasonic disintegrator in order to disperse clumped virus particles. Such treatment had no detectable effect on viral infectivity.

Hemagglutinin titrations. Saline was dispensed in 0.25-ml volumes from an automatic pipetting machine into "Prestware" plastic trays and twofold dilutions of virus made with a spiral loop (Takátsy, 1955). Fowl red blood cells (5%) were added as a drop (0.025 ml), and end points were determined by the pattern method. The standard deviation of a single titration was 0.03 $\log_{10}$ unit.

Infectivity titrations. Infectivity titrations were performed in fragments of allantois-on-shell (AOS) in plastic trays as described in detail elsewhere (Fazekas de St. Groth and White, 1958a).

Standard medium (SM). SM was prepared according to Fazekas de St. Groth and White (1958a), adjusted to pH 7.5 with NaOH, and autoclaved. The medium is not buffered, but rapidly equilibrates with the eggshell to pH 7.5. For long-term experiments (infectivity titrations) mycostatin was added at a concentration of 40 units per milliliter.

Reagents. Actinomycin D was a gift from Merck, Sharpe & Dohme Co., New Jersey. Amphotericin B ("Fungizone") and mycostatin ("Nystatin") were obtained from Squibb & Sons, New York; kanamycin sulphate ("Kanasig") from Sigma Co. Ltd., Melbourne, Australia; and DL-p-fluorophenylalanine (FPA) from Light and Co. Ltd., Colnbrook, England. Neuraminidase (RDE) was obtained from Dr. G. Ada as a semipurified preparation and was stored at −20°. It had a titer of 2000 as measured by the method of Burnet and Stone (1947). At a concentration of 100 units per milliliter the enzyme completely inhibited the uptake by AOS of 100 AD of BEL. No detectable regeneration of cell receptors occurred over the ensuing 8 hours.

Antisera. Rabbits were inoculated intravenously with 5 ml of BEL. A further 2.5 ml was emulsified with an equal volume of Freund's incomplete adjuvant and injected intramuscularly. Both injections were repeated at weekly intervals for 1 month, and the rabbits were then bled by cardiac puncture. After treatment with RDE the sera obtained had anti-HA titers of about 1000 against 5 AD of BEL.

Cell cultures. HeLa S3 cells were grown in monolayer culture in modified Eagle's medium (Eagle, 1959) containing 20% foetal calf serum. Cultures were fed twice weekly and passed weekly. BHK21, a diploid strain of hamster fibroblasts (Macpherson and Stoker, 1962), was propagated in medium 858 (Healy et al., 1955) with 20% foetal calf serum, and passed twice weekly. Both lines were kept free of mycoplasma by treatment every 2 months with 600 μg/ml of kanamycin

overnight. Cells were stored at $-65°$ in 10 % glycerol.

RESULTS

Measurement of Delay

Since the nature of delay is unknown, it is not yet possible to determine the time at which it begins or ends in any given cell. However, if all other steps in the viral multiplication cycle are of fairly constant duration, the extent of the delay can be determined by recording the time of occurrence of some subsequent phenomenon. This assumption was made in preliminary experiments designed to estimate the length of the delay in individual cells by measuring the time at which they developed the capacity to adsorb red blood cells.

Hemadsorption to Single Cells in Suspension

In HeLa cells influenza virus undergoes an abortive cycle of replication (Henle *et al.*, 1955). Even at very low m.o.i. no infectious virus is produced, hence infection does not proceed to a second cycle. Thus one can be confident that the late appearance of viral material in a particular cell truly reflects delay. Preliminary experiments confirmed that infection does not progress beyond a single cycle, but established that hemadsorption is demonstrable. There was no detectable increase in the yield of cell-associated hemagglutinin or in the number of hemadsorbing cells after 16–24 hours. Following infection at m.o.i. of less than 1, the final number of hemadsorbing cells was directly proportional to the input dose. At m.o.i. greater than 1, both cell-associated hemagglutinin and the number of hemadsorbing cells increased more quickly with time the higher the multiplicity.

Although infected cells could be satisfactorily identified by hemadsorption in monolayer cultures, noninfected cells proved more difficult to count. Moreover it was not possible to make sequential readings on a single culture. Accordingly a technique was developed which permitted hemadsorption to single cells in suspension (White *et al.*, 1962). "Mini-spinner" cultures, each of 10^6 cells suspended in 1 ml of modified Eagle's medium with 5 % foetal calf serum, were stirred

by a piece of paperclip sealed in glass capillary tubing and driven by a magnetic stirrer. Culture tubes were placed in circular racks in a plastic water bath which was constantly stirred and maintained at $36.5°$ (Fig. 1).

Cultures were inoculated with various doses of influenza, strain BEL. Thirty minutes later the cultures were centrifuged at $36.5°$ in the original tubes and washed in warm medium to remove unadsorbed virus. The tubes were then returned to the water bath and sampled at hourly intervals thereafter by removing 0.05 ml and mixing with an equal volume of 1 % fowl red blood cells. After 30 minutes at $4°$ each sample was transferred to a hemocytometer and counted. Hemadsorbing cells stood out clearly from the others (Fig. 2).

About 80 % of the cells were infectible, the remainder (perhaps undergoing mitosis) being refractory even to inocula of 100 ID_{50} per cell. The percentage of infectible cells hemadsorbing at each hour is plotted in Fig. 3.

It can be seen that at the highest m.o.i. most cells become hemadsorbing between the fourth and sixth hours post infection. However, as the m.o.i. decreases there is an increasing delay in the acquisition of this property by individual cells.

To demonstrate whether this phenomenon is qualitatively or quantitatively influenced by the particular type of cell used, the experiment was repeated in a nonmalignant, nonprimate, diploid cell strain of hamster fibroblasts, BHK21. Preliminary experiments in monolayer culture established that influenza virus, strain BEL undergoes an abortive cycle of multiplication in this cell strain also, and that synthesis of new viral material can be recognized by hemadsorption (Table 1).

Experiments using BEL in suspended BHK21 cells showed delay of an order similar to that seen with HeLa cells in suspension.

Differential Yields from AOS

To provide the precise quantitation required for more detailed analysis of this delay, the phenomenon was examined in AOS (Fazekas de St. Groth and White, 1958a). In this system forty to eighty pieces of allantois
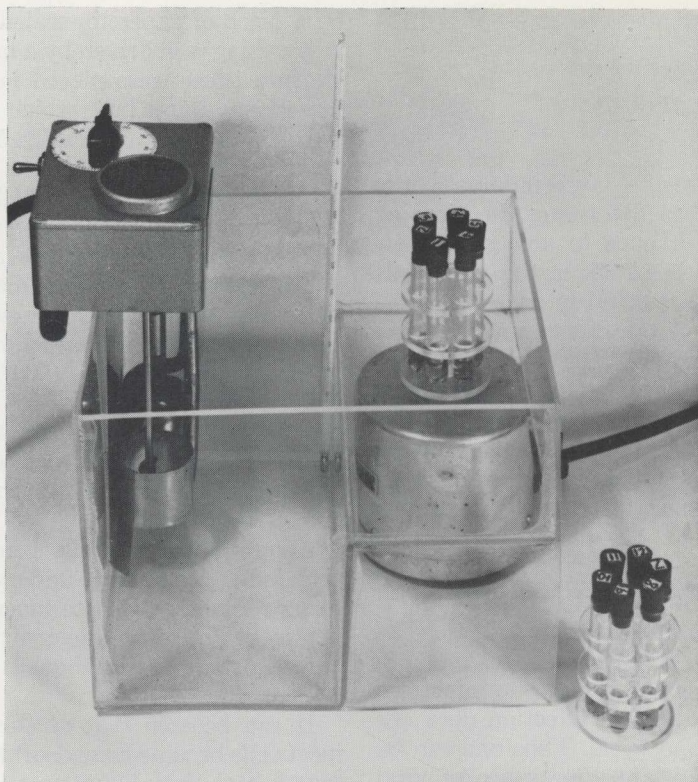
Fig. 1. Apparatus used for the maintenance of "mini-spinner" cultures.

still adherent to the shell are cut to a constant size from a single egg. All pieces of tissue are uniformly susceptible to infection and produce identical yields of virus (Fazekas de St. Groth and White, 1958b; White, 1959). Delay is demonstrable and is uninfluenced by cell susceptibility (White, 1960).

Squares of AOS from a 12-day-old egg were distributed into cups of a plastic tray containing 0.3 ml of prewarmed SM. After an hour's warming the tissue fragments were inoculated with various dilutions of BEL and the tray was placed on a shaking machine at 36.5°. All subsequent manipulations were conducted in a room maintained at 36.5°. After 15 minutes' shaking each cup received 100 units of prewarmed RDE, both to remove unpenetrated virus and to expedite the subsequent release of new virus from the cell surface (Cairns and Mason, 1953). Enzyme and virus were removed after a further 30 minutes, and the membranes were rinsed three times in warm SM with the aid of a suction apparatus and an automatic syringe.

At each hour thereafter the membranes were transferred to a fresh warm tray and the remaining supernatant fluids were titrated individually for their content of hemagglutinin. The standard error of the mean yield from four replicate membranes was $0.06 \log_{10}$ HA unit.

In Fig. 4 are plotted the quantities of virus newly produced in each hourly interval (differential yields) from groups of four replicate membranes at each of several m.o.i. The peak of each curve reflects the time at which virus is being produced at maximum rate, and the total virus yield is given by the integral of the curve.

Where the m.o.i. is far in excess of 1 the total yield is uninfluenced by the size of the inoculum; at m.o.i. below 1 the yield is proportional to the size of the inoculum. More important are the differences in shape of the curves. At high m.o.i. they rise steeply, more virus being released in the fourth hour than in any subsequent hour. As the m.o.i. decreases, so the maximum rate of virus

production occurs progressively later and the curves become flatter, until at m.o.i. of less than 1, virus is released over a long period with the peak of the curve occurring about 2 hours later.

The results are readily interpretable in terms of the mathematical model proposed by Cairns (1957). Virus is released from any individual cell according to a simple negative exponential function (Cairns, 1952). Under conditions of multiple infection random delays are overcome and all cells synthesize virus almost simultaneously; hence virus is released from the cell population according to a similar negative exponential function. Singly infected cells begin to synthesize virus after a delay, the duration of which varies from cell to cell; hence virus is released from the cell population more gradually with the maximum rate of release occurring later.

In numerous experiments with BEL in AOS the extent of the delay has been almost identical. Because of its convenience and reproducibility the system was used in the fol-

FIG. 3. Development by infected HeLa cells of the ability to adsorb red blood cells. The ordinate gives the percentage of infectible cells showing hemadsorption. Circles = m.o.i. 30; squares = m.o.i. 10; triangles = m.o.i. 3; inverted triangles = m.o.i. 1.

TABLE 1

MULTIPLICATION OF BEL IN BHK21 CELLS

Virus dilution	Percentage of cells hemadsorbing		
	1 day	2 days	3 days
$10^{-1.0}$	90	80	80
$10^{-1.5}$	80	60	50
$10^{-2.0}$	20	20	10
$10^{-2.5}$	3	3	1
$10^{-3.0}$	<1	<1	<1

lowing experiments designed to determine the stage or stages in the multiplication cycle at which delay can occur.

Delay in Adsorption and Penetration

In any system where the inoculum is never specifically removed, the continuing adsorption of virus will serve to increase the scatter in the time of initiation of viral replication in individual cells exposed to low m.o.i. The outcome will be similar if random delay occurs during penetration of the virus through the cell wall. Experiments with the enzyme neuraminidase, which halts the process of adsorption and retrieves all virus not already penetrated beyond a certain stage, did indeed confirm that such delays do occur.

FIG. 2. Hemadsorption to single HeLa cells in suspension. The hemocytometer field contains 3 positive and 4 negative cells.
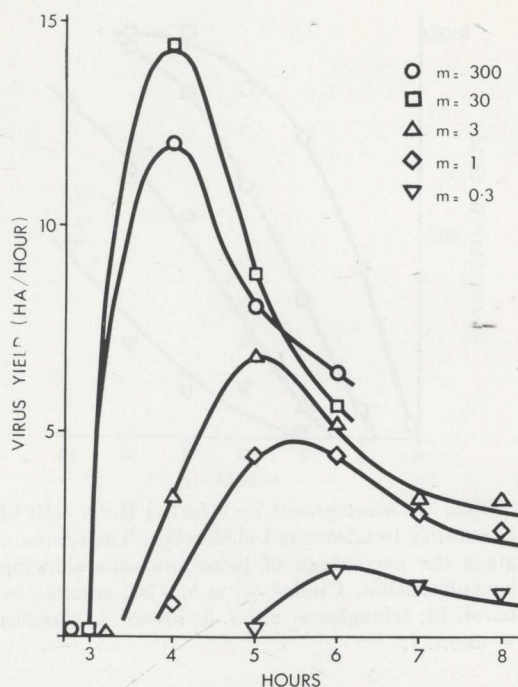
FIG. 4. Differential yields of hemagglutinin from AOS. BEL was inoculated at the various m.o.i. indicated on the graph, which shows the virus newly produced in each hourly interval.

When 100 units of RDE were added 100 minutes post infection the maximum rate of virus production from m.o.i. of <1 occurred $\frac{1}{2}$–$\frac{3}{4}$ hour later than if added 10 minutes p.i. Nevertheless, even when adsorption–penetration was restricted, the extent of the delay occurring at low m.o.i. was substantial (Fig. 4). Therefore the greater part of delay occurs after that stage of penetration characterized by the acquisition of resistance to neuraminidase. In all subsequent experiments the delays involved in adsorption and penetration were eliminated by the application of RDE at 10–15 minutes p.i. The enzyme serves an additional function. By releasing newly formed virus from the cell surface within 2 minutes of its completion (Cairns and Mason, 1953), RDE also eliminates any variable delay that may occur at this late stage.

Virus that has penetrated beyond the reach of RDE may still be accessible to antibody. To test whether delay occurs before or after the stage of antibody resistance, replicate pieces of AOS inoculated with BEL at a range of m.o.i. were treated 10 minutes p.i. with both RDE and BEL antiserum. The latter was used at a concentration previously shown to be nontoxic to the cells, yet capable, when added simultaneously with the virus, of preventing the growth of BEL at m.o.i. of 100. RDE and antiserum were removed at 40 minutes p.i. and differential yields of hemagglutinin were determined.

The curves obtained showed that delay is uninfluenced by the presence of antiserum and must thus occur after virus has penetrated beyond its reach.

Inhibition by p-Fluorophenylalanine

It has been clearly demonstrated that FPA inhibits the multiplication of influenza virus at two distinct points. When added late in the multiplication cycle it interferes with the synthesis of viral antigen (Ackermann and Maassab, 1955; Zimmermann and Schäfer, 1960; Scholtissek and Rott, 1961a,b); when when added at the beginning it inhibits the formation of "early proteins" required for the synthesis of viral nucleic acid as well as protein (Zimmermann and Schäfer, 1960; Scholtissek and Rott, 1961a,b). It has been demonstrated with poliovirus and Western equine encephalomyelitis virus (Wecker et al., 1962) that higher concentrations of FPA are required to block synthesis of "early proteins" than to block the subsequent synthesis of viral antigen.

If the process blocked by a particular concentration of FPA follows the stage at which delay occurs, then cells infected with single particles, hence showing a scatter of delays, might in the presence of the drug be permitted to "catch up" to those with no delay. On subsequent reversal of the inhibition, virus should then be produced synchronously in all cells. A preliminary experiment was carried out to select suitable concentrations of FPA. Dilutions of FPA were added to AOS half an hour before BEL at m.o.i. of 60. Five hours later the tissue was rinsed three times and phenylalanine (PA) was added to each cup at a concentration of 100 μg/ml to reverse the block. Cumulative yields of hemagglutinin were determined at each hour thereafter. Yields from untreated controls were titrated from 3 hours onwards (Fig. 5).

There are striking differences in the times at which new virus appeared after the inhibition by different doses of FPA was reversed. AOS receiving concentrations of 200, 400, or 800 μg/ml began to release new virus 3–3½ hours after removal of the FPA. Since the FPA was present for 5 hours and virus was first released from uninhibited control cultures at 3 hours, these high doses of FPA must block a process that occurs very shortly after infection. On the other hand, AOS treated with concentrations of 50 or 100 μg/ml released new virus 1½ hours after removal of the drug, indicating that they block some 1½–2 hours later than the higher doses, i.e., at about 1½–2 hours p.i. This corresponds with the time at which the synthesis of influenza A (fowl plague) structural protein is known to begin (Scholtissek and Rott, 1961b; Schäfer, 1963). Concentrations of less than 50 μg/ml only partially inhibit the multiplication of this dose of virus.

Synchronization of Viral Multiplication by FPA

FPA was added to AOS at a concentration of 100 μg/ml, then BEL was inoculated at a range of m.o.i. 30 minutes later. At 5 hours p.i. the FPA was rinsed out and PA was substituted. At hourly intervals thereafter the differential yields were titrated, a corresponding control set being assayed from 3 hours p.i. (Fig. 6).

New virus began to appear 2 hours after removal of this dose of FPA. Virus was produced more slowly and over a longer period from the FPA-treated tissue than from the untreated controls, shown on the left. However, the striking feature of the FPA curves is their similarity to one another. They do not show any of the delay so clearly evident in the controls. Delay has been overcome by blocking viral structural protein synthesis with FPA at low concentration. It can be concluded that delay occurs before the synthesis of new viral structural protein.

Similar experiments with high doses of FPA gave erratic results that were difficult to interpret. However, the following approach to the same problem proved more productive.

Inhibition by Late Addition of FPA

AOS was inoculated with BEL at m.o.i. of 1 or 100, and FPA (600 μg/ml) was added at intervals thereafter. Total yields of hemagglutinin after 8 hours were titrated and expressed as a percentage of the 8-hour yield from uninhibited controls (Fig. 7).

High concentrations of FPA have been shown (Fig. 5) to block multiplication very shortly after infection but if added later will
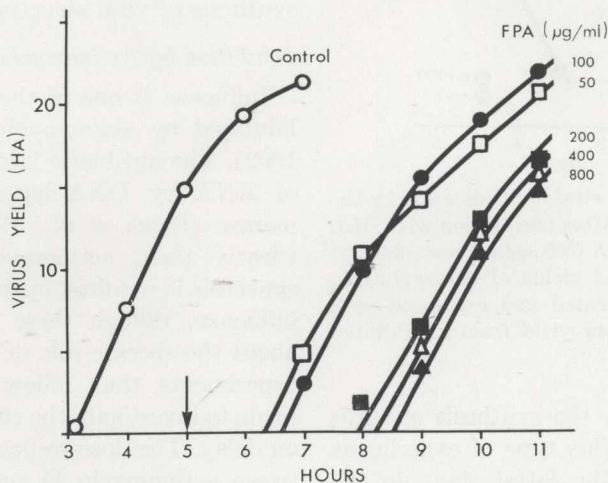

FIG. 5. Time of action of FPA. AOS was inoculated with BEL at m.o.i. of 60 in the presence of various concentrations of FPA. The drug was removed 5 hours p.i. and the cumulative yields of hemagglutinin were titrated at hourly intervals thereafter.
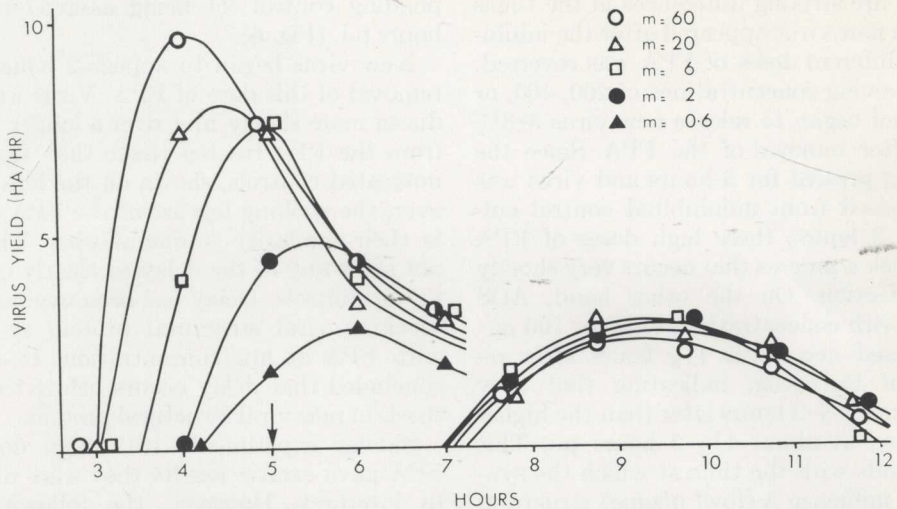
FIG. 6. Differential yields of hemagglutinin following inhibition by FPA. AOS was inoculated with BEL at various m.o.i. in the presence of FPA (100 μg/ml). The drug was removed 5 hours post infection and the hemagglutinin newly produced in each hourly interval was titrated. Differential yields from untreated AOS are shown on the left.
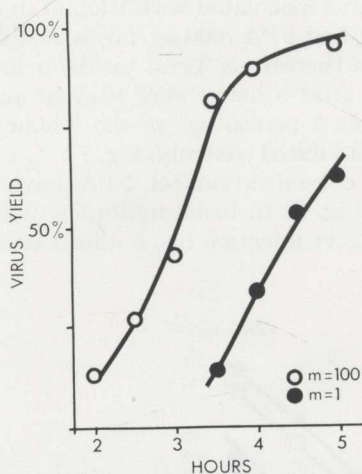

FIG. 7. Inhibition of viral multiplication by the late addition of FPA. After inoculation with BEL at m.o.i. of 1 or 100, FPA (600 μg/ml) was added at the times shown. Total yields of hemagglutinin after 8 hours were titrated and expressed as a percentage of the 8-hour yield from uninhibited controls.

obviously also block the synthesis of virus structural protein. This type of experiment therefore indicates the latest stage in the viral multiplication cycle that FPA is capable of blocking. Hence it may be seen from

Fig. 7 that even the high dose of virus is still inhibited by FPA added as late as 2 hours p.i. The significant finding is the difference between the high and low multiplicity curves. Virus at low multiplicity is inhibited about 1½ hours later than at high, the difference between the two curves reflecting the difference in extent of the delay shown at high and low m.o.i. The fact that this delay is still demonstrable indicates that it precedes the synthesis of viral structural protein.

Inhibition by Actinomycin D

Influenza is one of the few RNA viruses inhibited by actinomycin D (Barry et al., 1962). The antibiotic inhibits the formation of RNA by DNA-dependent RNA polymerase (Reich et al., 1962; Reich, 1964). Clearly then, actinomycin-sensitive RNA synthesis is required in the multiplication of influenza, though there is no information about the specific role of such RNA. In the experiments that follow an attempt was made to investigate the effect of actinomycin on delay. The dose-response relationship between actinomycin D and BEL was determined by adding various concentrations of the drug to AOS 30 minutes before a range of
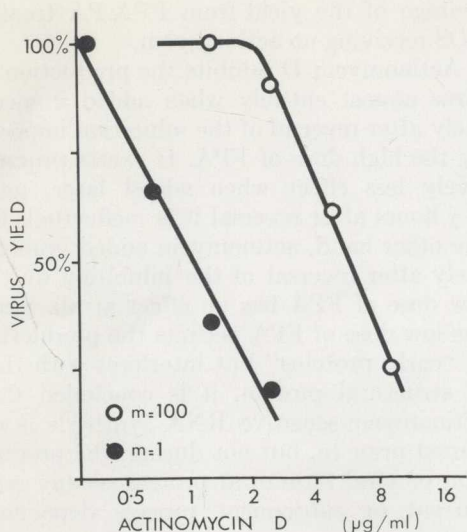
FIG. 8. Effect of actinomycin D on the production of BEL. AOS was infected at m.o.i. of 1 or 100 in the presence of various concentrations of actinomycin and the yield of hemagglutinin was titrated 8 hours later.
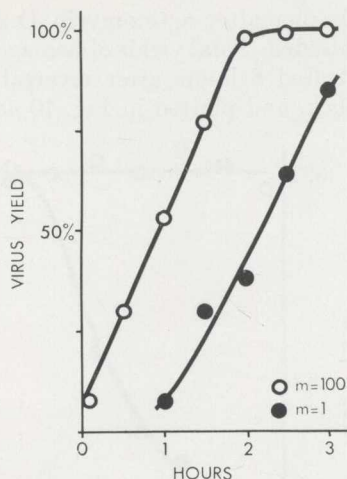

FIG. 9. Inhibition of viral multiplication by the late addition of actinomycin D. After inoculation with BEL at m.o.i. of 1 or 100, actinomycin D (20 µg/ml) was added at the times shown. Total yields of hemagglutinin after 8 hours were titrated and expressed as a percentage of the 8-hour yield from uninhibited controls.

multiplicities of BEL and titrating total yields of hemagglutinin 8 hours p.i. (Fig. 8).

The dose response curves are quite steep, but higher concentrations of the drug are required to inhibit the higher concentrations of virus. Unpublished experiments revealed that at the concentrations used actinomycin D could not be removed from this system by washing. Indeed within 20–30 minutes 50% of the added drug was already irreversibly bound.

Inhibition by Late Addition of Actinomycin D

The irreversibility of action of actinomycin D ruled out the possibility of trying to synchronize viral multiplication by prolonged application of the drug. However, it seemed feasible to use the alternative approach, i.e., to add the drug at successively later times after infection and titrate the 8-hour yield. The experimental design was as described for FPA, using actinomycin D at 20 µg/ml. Fig. 9 shows the results.

The figure resembles that obtained with FPA, the low m.o.i. curve falling about 1¼ hours to the right of the other. This reflects a delay of about the same degree as before and

indicates that such delay occurs prior to the time of action of actinomycin D.

Mode of Action of Actinomycin D

It is difficult to interpret this finding in terms of some particular stage in the cycle, for the mechanism of inhibition of influenza virus multiplication by actinomycin is still unknown. However, it is clear from Fig. 9 that actinomycin inhibits only early in the cycle. Cells infected at high m.o.i., hence showing minimum delay, produce a yield fully 50% that of untreated controls when the drug is added at 1 hour p.i., while actinomycin added at 2 hours p.i. has no inhibitory effect at all. It would appear that, once a critical step has been completed, the antibiotic cannot block the synthesis of viral RNA and structural protein, neither of which begins until about 2 hours p.i. (Scholtissek and Rott, 1961b; Schafer, 1963) (Figs. 5 and 7). This conclusion is supported by the result of the following experiment.

FPA (100 or 600 µg/ml) was added to AOS 30 minutes before BEL (m.o.i. = 50). Residual inoculum was removed with RDE, but the FPA was left in for 5 hours p.i. before being rinsed out and replaced by PA. At

intervals thereafter actinomycin D (20 μg/ml) was added. Total yields of hemagglutinin were titrated 8 hours after reversal of the FPA block, and plotted in Fig. 10 as a per-
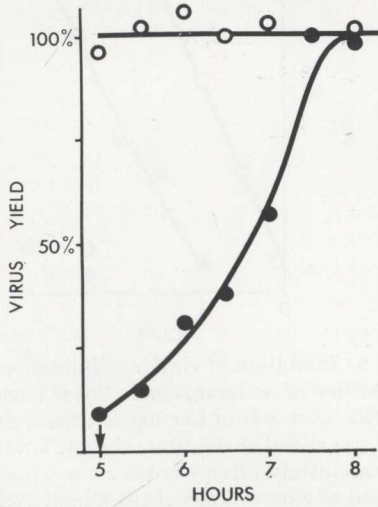

Fig. 10. Relative times of action of FPA and actinomycin D. AOS was inoculated with BEL at m.o.i. of 50 in the presence of FPA at 100 μg/ml (open circles) or 600 μg/ml (closed circles). Five hours later the FPA was removed and replaced with PA. Actinomycin D (20 μg/ml) was then added at the times shown. Total yields of hemagglutinin were titrated 8 hours after reversal of the FPA block, and plotted as a percentage of the yield from FPA-PA treated membranes receiving no actinomycin.

centage of the yield from FPA-PA treated AOS receiving no actinomycin.

Actinomycin D inhibits the production of virus almost entirely when added immediately after reversal of the inhibition imposed by the high dose of FPA. It exerts progressively less effect when added later, until $2\frac{1}{2}$ hours after reversal it is ineffectual. On the other hand, actinomycin added immediately after reversal of the inhibition by the low dose of FPA has no effect at all. Since the low dose of FPA permits the production of "early proteins" but interferes with that of structural protein, it is concluded that actinomycin-sensitive RNA synthesis is required prior to, but not during, the production of viral structural protein or any concurrent or subsequent process dependent upon it.

The same conclusion may be reached from a different direction. The experiments leading to Figs. 7 and 9 were repeated as a single experiment in which AOS inoculated with BEL at m.o.i. of 100 received FPA (600 μg/ml) or actinomycin D (20 μg/ml) at intervals after infection. Total 8-hour yields are plotted in Fig. 11 as a percentage of untreated controls.

At such high m.o.i. the results are not complicated by the occurrence of delay. The position of the curves indicates the latest stage in the multiplication cycle at which
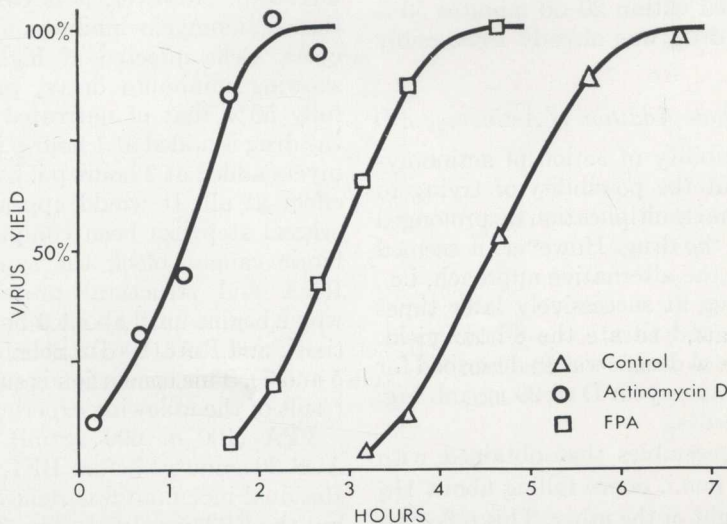

Fig. 11. Relative times of action of FPA and actinomycin D. After inoculation with BEL at m.o.i. of 100, FPA (600 μg/ml) or actinomycin D (20 μg/ml) was added at the times shown. Total yields of hemagglutinin after 8 hours were titrated and expressed as a percentage of the 8-hour yield from uninhibited controls. Another set of controls was sampled from 3 hours onwards and expressed in the same way.

each of the drugs is able to act. Actinomycin D inhibits a process (or processes) that begins almost immediately p.i., is 50% complete at 1 hour and is over within the first 2 hours of the multiplication cycle. FPA inhibits the production of functional viral structural protein, beginning about $1\frac{3}{4}$ hours p.i. An average of $1\frac{1}{2}$–2 hours elapses between the synthesis of a protein molecule and its emergence from the cell in a mature virus particle.

DISCUSSION

Though the emphasis in this paper has been on delay, the experiments involving the action of inhibitors on cells infected at high m.o.i. throw some light on a number of other aspects of influenza virus multiplication.

Fluorophenylalanine acts at two quite separate points in the multiplication cycle. High concentrations block very shortly after infection, whereas lower doses exert their effect $1\frac{1}{2}$–2 hours later (Fig. 5). The first corresponds to the inhibition of synthesis of "early proteins," and the second to the inhibition of synthesis of viral structural protein, as demonstrated in other ways by previous workers (Zimmermann and Schäfer, 1960; Scholtissek and Rott, 1961a, b). The synchronization of viral production following inhibition with a low dose of FPA (Fig. 6) and the effectiveness of FPA in blocking low m.o.i. up to $1\frac{1}{2}$ hours later than high (Fig. 7) provided clear evidence that delay occurs before the commencement of synthesis of viral structural proteins. The marked flattening of all the synchronized curves may simply be due to a generalized slowing down of cell metabolism, hence of virus production, following prolonged drug treatment of cells already barely subsisting in a very simple maintenance medium.

Actinomycin D blocks an early stage of the influenza viral multiplication cycle (Fig. 9). By the time the synthesis of viral structural protein begins actinomycin D is totally ineffective. It is concluded that no cell-coded messenger RNA synthesis is required during the synthesis of viral structural protein. This conclusion is borne out by the finding that actinomycin is totally ineffective when added immediately after reversal of a 5-hour inhibition of synthesis of viral structural protein with a low concentration of FPA (Fig. 10). On the other hand, virus production is almost completely blocked when actinomycin is added immediately after reversal of a high concentration of FPA. There are a number of possible interpretations of this finding. For example, it might be supposed that the first cell-coded messenger RNA involved in viral multiplication is unstable, but this appears unlikely in view of the demonstrable longevity of mammalian cell messenger RNA (Reich et al., 1962). Alternatively, if DNA-dependent RNA polymerase ("transcriptase") were turned over rapidly in the virus-infected cell, all the enzyme synthesized in the presence of a high concentration of FPA could be expected to incorporate the analogue in place of PA, hence be nonfunctional (Munier and Cohen, 1959). This too seems improbable in the light of the reported stability of transcriptase (Holland, 1963). The simplest and most probable explanation of the findings of Fig. 10 is that the first process to be blocked by actinomycin follows the first process blocked by FPA (600 μg/ml). This would mean that the first protein required for viral multiplication is coded by the viral RNA, not by the host cell DNA. As a corollary it could be concluded that the incoming virus particle is uncoated without the participation of host-coded messenger RNA, i.e., without induction of any host-coded uncoating enzyme. Viral nucleic acid would presumably be released by a nonenzymatic process or via the action of enzymes already present in adequate concentration in the cell. Clearly all those RNA viruses that can multiply in the presence of actinomycin (Reich et al., 1962) must be uncoated in some such way, although the second stage of uncoating of vaccinia virus has been clearly shown to involve the induction of an enzyme coded by the cell genome but normally repressed in the uninfected cell (Joklik, 1962, 1964a, b). Some unpublished experiments may be significant in this regard. Delay in the multiplication of low m.o.i. of influenza virus could not be abolished by 2 hours' preinfection or simultaneous infection with high m.o.i. of homologous incomplete virus or homologous virus irradiated with any of a wide variety of doses of ultraviolet light. It is noteworthy too that

Cords and Holland (1964) could not abolish the "lag" in synthesis of poliovirus RNA by preinfecting the cells with polio in the presence of guanidine, even though eclipse occurs normally in the presence of this drug.

If actinomycin does act on some stage subsequent to uncoating, two possible points of action can be suggested. The antibiotic may block the synthesis on cellular DNA of a complementary RNA which acts as a messenger(s) for synthesis of protein(s) required for the execution of some step subsequent to uncoating. Alternatively, influenza viral RNA may be converted to a temporary double-stranded form which then serves as a template for the synthesis of viral RNA, and such synthesis may be inhibited by actinomycin. This could possibly explain the recent finding (Rott and Scholtissek, 1964) that actinomycin added $1\frac{1}{2}$–2 hours p.i. stops the synthesis of fowl plague RNA while permitting the continued production of S-antigen. However, their results may equally suggest that viral RNA replication is catalyzed by an RNA-dependent RNA polymerase ("replicase") which is unstable like that described by Baltimore et al. (1963) but cell-coded, hence not synthesized in the presence of actinomycin. In this connection however, it must be remembered that actinomycin does not affect the replication of other single-stranded RNA viruses for which double-stranded intracellular RNA has been reported (Montagnier and Sanders, 1963; Baltimore, 1964). In such cases it has been shown that replication of viral RNA is carried out by a novel enzyme, RNA-dependent RNA polymerase ("replicase") that is coded by the viral RNA itself and is not inhibited by actinomycin (Baltimore and Franklin, 1963). It is interesting that actinomycin blocks the multiplication of reovirus, the RNA of which is double-stranded (Gomatos et al., 1962).

There is much confusion in the literature about the meaning and interpretation of "delay" and "asynchrony." Clearly, there are many steps in the viral multiplication cycle that occupy a finite but constant length of time. However "delay," in the sense used in this study, refers only to those delays of variable duration determined by m.o.i. Such delays may occur at one or more stages in the viral multiplication cycle. For example, because adsorption proceeds exponentially with time, multiplicity-dependent delays are demonstrable in the processes involved in adsorption-penetration. Since the multiplication cycle is not deemed to have begun until adsorption has occurred, such delay is largely irrelevant. In the experiments reported they were minimized by the application of RDE 10–15 minutes p.i. The major part of delay was found to follow those stages of penetration at which virus is still accessible to neuraminidase or antibody. Yet this delay precedes the synthesis of viral structural protein (Figs. 6 and 7) and also precedes the point of action of actinomycin D (Fig. 9). Within these limits it is possible to envisage a number of stages at which such delay could conceivably occur. For example, random delay could take place while the incoming virus particle is still inside its pinocytotic vesicle. The extent of the delay might represent the time elapsing before the relevant enzymes succeed in gaining access to the virus, e.g., the probability that a lysosome releases its contents into the vesicle in unit time. Delay could be viewed essentially as a topographical phenomenon. Combination of the uncoated viral NA molecule with an unprogrammed ribosome may be an event with a low probability of occurrence in unit time. One infecting viral RNA molecule must presumably complete with a vast excess of normal cell messenger RNA being synthesized continuously. The chance that one viral NA molecule succeeds in attaching to a free ribosome would be proportional to the m.o.i. At this stage there might also be a finite probability that a viral NA molecule becomes inactivated by nucleases. This may be an important cause of low "plating efficiency" of viruses (i.e., high cellular resistance) in those many instances where adsorption, penetration, capacity of the cell to support virus multiplication from high m.o.i., and yield of new virus from high m.o.i. are all demonstrably normal (e.g., White, 1959). In a sense delay could then be considered as infinite.

In studies of the sort recorded in Fig. 11, where the time of action of an inhibitor is

determined by the sequential addition of the drug at intervals post infection, it is worth recalling that the drug will always appear to act somewhat earlier than it actually does because of the finite time it requires to penetrate to its site of action. Such errors can be minimized by using the highest practicable concentration of the inhibitor. Such was the case in the experiments summarized in Figs. 7, 9, and 11, where a three-fold reduction in the dose of drug employed made no detectable difference to the position of the curves. Nevertheless, allowing for the known penetration time of these inhibitors, estimates of their time of action may be up to $\frac{1}{4}$ hour out. Within these limits it has been demonstrated (Fig. 11) that actinomycin D and FPA (600 $\mu g/ml$) block events occurring $1\frac{1}{2}$–2 hours before the synthesis of viral structural protein begins and that a further $1\frac{1}{2}$–2 hours elapses before that protein appears extracellularly in the form of mature virus particles.

It would be interesting to know whether all viruses show delay in their multiplication cycles. The phenomenon has so far been clearly demonstrated with three very different viruses, influenza (Cairns, 1957), vaccinia (Cairns, 1960), and poliovirus (Cooper, in press), while isolated observations in the literature suggest that it may comprise an integral part of the reproductive cycle of several other animal viruses.

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2

Early Events in the Eclipse Phase of Influenza and Parainfluenza Virus Infection[1]

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The role of the cell nucleus in the multiplication of influenza and parainfluenza viruses has been examined. While the capacity of cells to support the growth of parainfluenza virus is relatively resistant to preirradiation with ultraviolet light, their capacity to support the growth of influenza virus is just as sensitive as the cell-virus complex shortly after infection. Between $\frac{1}{2}$ and $2\frac{1}{2}$ hours post-infection the cell-virus complex acquires resistance to irradiation. The findings indicate that cell-coded messenger RNA is required in the early stages of influenza virus multiplication, but plays no role in the replication of viral nucleic acid or any subsequent event.

Multiplicity-dependent delay in the multiplication of influenza virus takes place after the synthesis of at least one "early" protein but before the latest point of action of actinomycin D, before the acquisition of resistance to ultraviolet irradiation and before the establishment of interference.

INTRODUCTION

There is good evidence that influenza and parainfluenza viruses differ in some very fundamental way in their modes of replication (Waterson, 1962; Barry, 1964a,b). Newcastle disease virus (NDV), a parainfluenza-like virus, will multiply in the presence of actinomycin D (Barry *et al.*, 1962) or mitomycin C (Rott *et al.*, 1965), or after pretreatment of the host cells with high doses of ultraviolet light (Rubin and Temin, 1959; Barry, 1964a,b). On the other hand, the multiplication of fowl plague, an influenza virus, is inhibited by actinomycin D (Barry *et al.*, 1962), mitomycin C (Rott *et al.*, 1965) or pretreatment of the host cells with relatively low doses of ultraviolet light (Barry, 1964a,b). The findings with NDV are widely accepted to mean that cell DNA is in no way essential to the replication of that virus. Indeed, inhibition of cell messenger RNA synthesis by actinomycin D, or removal of cell messenger RNA from polyribosomes by

brief treatment with puromycin, actually increases the rate of parainfluenza virus multiplication (White and Cheyne, 1965). However, there is some difference of opinion about the interpretation of the findings with influenza, particularly with respect to actinomycin D. Barry (1964a,b) suggests that they indicate a vital role for cell-coded information in the multiplication of influenza virus, but also raises the possibility that actinomycin may be acting more directly as an inhibitor of viral RNA replication by binding to a hypothetical double-stranded RNA template. In this regard Dulbecco (1965) has suggested that the influenza RNA-dependent RNA polymerase may be more sensitive to actinomycin than those of the viruses know to produce a double-stranded "replicative" RNA, all of which are resistant to the drug (Montagnier and Sanders, 1963; Baltimore, 1964). Then again, Rott *et al.*, (1965) argued that actinomycin may well be acting by actually destroying the viral RNA in the cell nucleus. There is yet another possibility put forward orignally with respect to Rous sarcoma virus by Temin (1964), that actinomycin may bind to a novel DNA, which

has been synthesized *de novo* from a template of viral RNA. Despite all these alternatives, there is strong evidence to support the view that the virus-inhibitory action of actinomycin is primarily directed at nuclear DNA. Firstly, maximum inhibition of influenza virus multiplication is demonstrable in cells pretreated for half an hour with actinomycin but washed free of the drug before infection (White *et al.*, 1965). Within this interval the drug has become irreversibly bound, presumably to cell DNA. Secondly, though the multiplication of influenza virus at high multiplicity is blocked by actinomycin added at the time of infection, the antibiotic is totally ineffective if added 2 hours after infection (White *et al.*, 1965). Since influenza viral RNA synthesis does not begin until 2 hours (Scholtissek and Rott, 1961; Schäfer, 1963), the inhibitory action of actinomycin can hardly be attributed to its binding to any double-stranded replicative form of viral RNA. The observation of Rott *et al.* (1965) that actinomycin can in fact block the multiplication of fowl plague virus even when added after viral RNA synthesis has begun, provided that an additional 4 hours be allowed for the drug to take effect, could equally be interpreted to mean that the drug is inhibiting the synthesis of a cell-coded messenger RNA that is continuously required, begins to be produced early in the eclipse period, and has a half-life of a few hours. The experiments described in the first half of this paper, employing ultraviolet irradiation in place of actinomycin, may resolve this issue. They indicate that the cell nucleus does supply information vital to the growth of influenza virus, but that the need for it is over quite early in the multiplication cycle.

The second part of the paper deals with multiplicity-dependent delay in the multiplication of influenza virus (Cairns, 1957). It has previously been shown that this delay occurs after the virus has penetrated beyond the reach of antibody, but before the synthesis of viral structural protein, and before the point of action of actinomycin D (White *et al.*, 1965). The experiments that follow reveal that delay takes place after the synthesis of "early" protein(s), but before the

establishment of interference, and before the acquisition of resistance to ultraviolet light.

MATERIALS AND METHODS

Viruses. The BEL strain of influenza type A, the Swine (SW) strain of influenza type A, and the Sendai strain of parainfluenza type 1 were all grown in the allantoic cavity of embryonated eggs and stored at $-65°$. Before each experiment virus was treated for 60 seconds in an M.S.E. 500-watt ultrasonic disintegrator to disperse any clumped particles.

Infectivity titrations. Infectivity titrations were carried out in fragments of allantoic membrane suspended in Standard Medium in plastic trays. The details have been given elsewhere (Fazekas de St. Groth and White, 1958; White *et al.*, 1965). As all the experiments described in this paper were conducted in this system, "multiplicity of infection" (m.o.i.) is taken as the number of infectious units adsorbed per cell.

Hemagglutination titrations. The procedure has been described elsewhere (White *et al.*, 1965). Because of its simplicity and precision this technique was routinely employed to measure the amount of virus released into the supernatant fluid from allantoic cells maintained *in vitro*. In this regard it must be borne in mind that most of the virus expelled from these cells under conditions of multiple infection is incomplete. The hemagglutination technique does not discriminate between complete and incomplete virus, but records the total number of particles released. Following infection of this system at high m.o.i., new virus begins to appear in the supernatant fluid after a latent period of 3 hours for influenza strains BEL or SW (White *et al.*, 1965) and $6\frac{1}{2}$–7 hours for the parainfluenza strain Sendai (White and Cheyne, 1965).

Titrations of mixed yields of BEL and SW were conducted in the presence of SW or BEL antiserum, prepared according to White *et al.*, (1965). Concentrations of antisera were selected at which no inhibition of hemagglutination by the heterologous virus was demonstrable. When mixed yields were titrated in the presence of BEL antiserum, then separately in the presence of SW anti-

serum, the total yield of virus was equal to that measured in the absence of either serum. If any phenotypic mixing occurred, it was quantitatively too little to be detected.

Ultraviolet irradiation. Irradiation was carried out with a 15-watt General Electric Germicidal lamp. Membranes were rinsed in 0.9% NaCl, drained, and immediately irradiated in an open petri dish at 63 cm. At this distance the intensity of radiation was about 10 ergs/mm²/second.

RESULTS

Development of Resistance to Ultraviolet Light by Parainfluenza Virus-Infected Cells

Plastic trays containing uniform squares of allantois-on-shell cut from a single egg and suspended in Standard Medium were agitated on a horizontal shaking machine at 36° for 1 hour. Sendai virus was then inoculated as a single drop to provide a multiplicity of infection (m.o.i.) of 1 or 100. After 30 minutes 100 units of neuraminidase were added to remove virus that had not yet penetrated the cells. A further 30 minutes later residual virus and neuraminidase were removed together and the cells were rinsed then transferred to a fresh prewarmed tray of medium. At half an hour before, half an hour after, and 5 hours after infection, groups of membranes were removed, rinsed in 0.9% NaCl and immediately irradiated in an empty petri dish with a range of doses of ultraviolet light (UVL). The membranes were then returned to another prewarmed tray of medium and incubation was continued. Eighteen hours post-infection (p.i.)
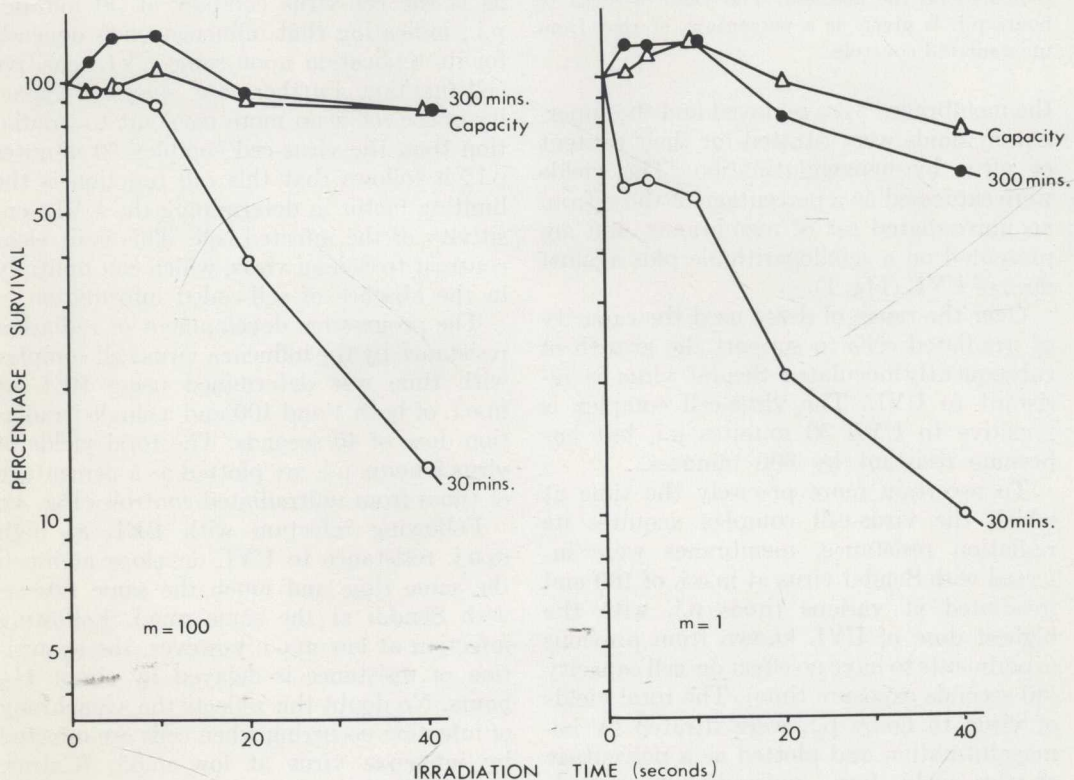

FIG. 1. Development of resistance to ultraviolet light by parainfluenza virus-infected cells. Membranes were irradiated for the number of seconds indicated on the abscissa at 30 minutes before infection ("capacity"), 30 minutes after infection, and 300 minutes after infection with Sendai virus at m.o.i. of 100 (left) or 1 (right). The yield of virus 18 hours p.i. is given on a logarithmic scale as a percentage of that from unirradiated controls.
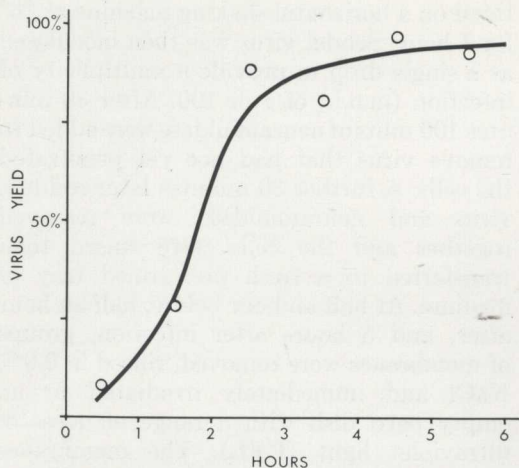
FIG. 2. Development of resistance to ultraviolet light by parainfluenza virus-infected cells. Membranes infected with Sendai at m.o.i. of 100 were irradiated for 40 seconds at the time p.i. indicated on the abscissa. The yield of virus 18 hours p.i. is given as a percentage of that from unirradiated controls.

the membranes were removed and the supernatant fluids were titrated for their content of virus by hemagglutination. The yields were expressed as a percentage of those from an unirradiated set of membranes, and are presented on a semilogarithmic plot against dose of UVL (Fig. 1).

Over the range of doses used the capacity of irradiated cells to support the growth of subsequently inoculated Sendai virus is resistant to UVL. The virus-cell complex is sensitive to UVL 30 minutes p.i, but has become resistant by 300 minutes.

To ascertain more precisely the time at which the virus-cell complex acquires its radiation resistance, membranes were infected with Sendai virus at m.o.i. of 100 and irradiated at various times p.i. with the highest dose of UVL known from previous experiments to have no effect on cell capacity (40 seconds exposure time). The total yields of virus 18 hours p.i. were titrated by hemagglutination and plotted as a percentage of the yield from unirradiated controls (Fig. 2). Radiation resistance begins to develop as early as ½ hour p.i., then rises sharply after 1½ hours to reach by 2½ hours 80 % of the control level.

Development of Resistance to Ultraviolet Light by Influenza Virus-Infected Cells

Similar experiments were conducted with influenza virus (strain BEL). Membranes infected at m.o.i. of 1 or 100 were irradiated with various doses of UVL half an hour before, half an hour after and 2½ hours after infection. Total yields were titrated at 8 hours p.i. (Fig. 3).

Again there is a marked difference in the radiation resistance of cell-virus complexes irradiated early and late in the latent period, this difference being more pronounced at high m.o.i. The most striking finding, however, is that the capacity of uninfected cells to support the multiplication of subsequently inoculated influenza virus is much more sensitive to irradiation than the capacity to support the multiplication of Sendai. Indeed, the capacity is precisely as sensitive as is the cell-virus complex at 30 minutes p.i., indicating that influenza virus depends for its replication upon some UVL sensitive cell function. Furthermore, since the capacity of the cell is no more resistant to irradiation than the virus-cell complex 30 minutes p.i., it follows that this cell function is the limiting factor in determining the UVL sensitivity of the infected cell. This is in clear contrast to Sendai virus, which can multiply in the absence of cell-coded information.

The progressive development of radiation resistance by the influenza virus-cell complex with time was determined using BEL at m.o.i. of both 1 and 100 and a single irradiation dose of 40 seconds. The total yields of virus 8 hours p.i. are plotted as a percentage of those from unirradiated controls (Fig. 4).

Following infection with BEL at high m.o.i. resistance to UVL develops at much the same time and much the same rate as with Sendai at the same m.o.i. Following infection at low m.o.i. however, the acquisition of resistance is delayed by about 1½ hours. No doubt this reflects the asynchrony of infection occurring when cells are infected by influenza virus at low m.o.i. (Cairns, 1957). Multiplication of virus in such cells is delayed by an average of 1½ hours at a point somewhere between the penetration of the virus into the cell and an actinomycin-sensitive step (White et al., 1965). Since the
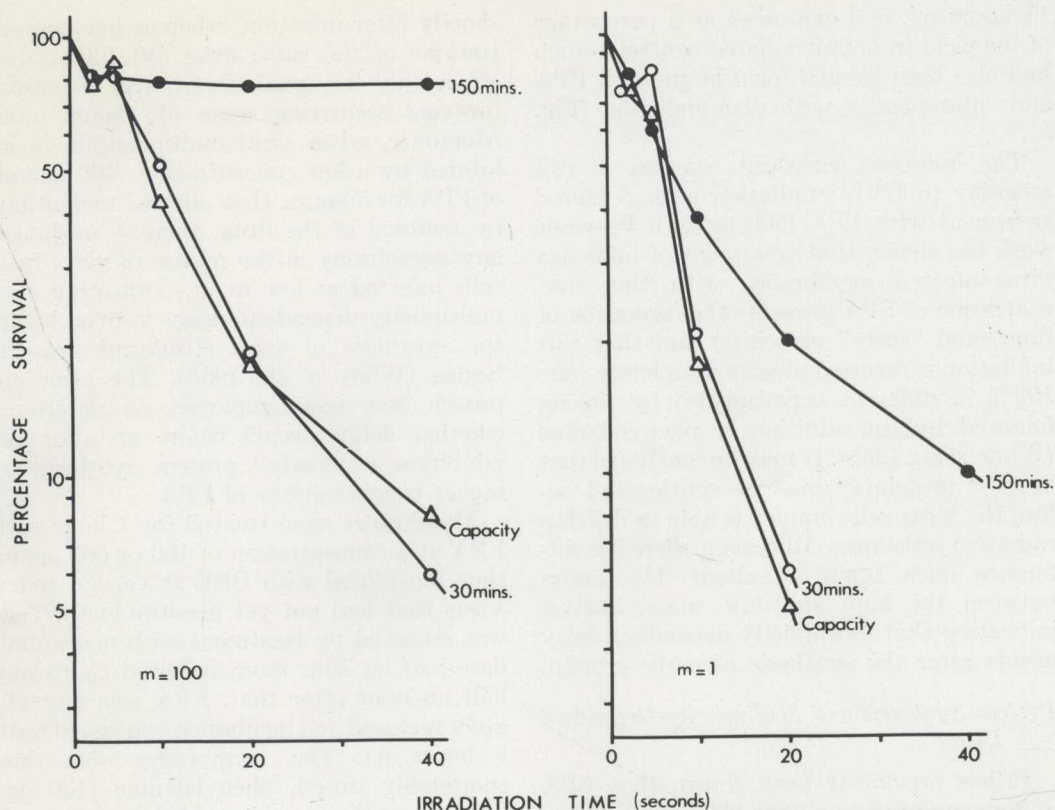
Fig. 3. Development of resistance to ultraviolet light by influenza virus-infected cells. Membranes were irradiated for the number of seconds indicated on the abscissa at 30 minutes before infection ("capacity"), 30 minutes after infection, and 150 minutes after infection with BEL virus at m.o.i. of 100 (left) or 1 (right). The yield of virus 8 hours p.i. is given on a logarithmic scale as a percentage of that from unirradiated controls.

same multiplicity-dependent delay is apparent in Fig. 4, it is now clear that this delay occurs at some stage prior to the acquisition of radiation resistance by the influenza virus-cell complex. On looking back at Fig. 3, it will be seen that the same phenomenon is apparent there. By 150 minutes p.i. cells infected at m.o.i. of 100 have become fully resistant to the doses of UVL used, whereas those infected at m.o.i. of 1 are still only partially resistant because the transition has been delayed in a proportion of those cells.

Protein Synthesis and Development of Radiation Resistance

The next experiment was designed to determine the relationship in time between multiplicity-dependent delay, development of radiation resistance and the synthesis of the first proteins required in influenza virus multiplication. Membranes were treated with p-fluorophenylalanine (FPA) at a concentration of 600 μg/ml for 1 hour at 36°, then inoculated with BEL at m.o.i. of 1 or 100. Neuraminidase was added 30 minutes later and removed, together with residual virus, 30 minutes after that. The membranes were rinsed and quickly transferred into another prewarmed tray containing FPA (600 μg/ml). At 5 hours p.i. the FPA was removed, and the membranes rinsed then placed in a fresh tray of medium containing phenylalanine at a concentration of 100 μg/ml. At intervals, groups of membranes were irradiated for 40 seconds then replaced in the tray for further incubation. Total yields from each membrane were titrated at

11 hours p.i. and expressed as a percentage of the yield from unirradiated controls which had also been treated for 5 hours with FPA and subsequently with phenylalanine (Fig. 5).

The influenza virus-cell complex is still sensitive to UVL irradiation after 5 hours' treatment with FPA (600 μg/ml). Previous work has shown that treatment of influenza virus-infected membranes with this concentration of FPA prevents the formation of functional "early" protein(s) and that this inhibition is reversed almost completely (50–100% in different experiments) by rinsing followed by the addition of phenylalanine (White et al., 1965). It may be concluded that "early" protein(s) must be synthesized before the virus-cell complex is able to develop radiation resistance. Moreover, there is a difference once again of about 1½ hours between the high and low m.o.i. curves, indicating that multiplicity-dependent delay occurs after the synthesis of early protein.

Protein Synthesis and Multiplicity-Dependent Delay

It has previously been shown that FPA at high concentration (200–800 μg/ml) interferes with the synthesis of "early" protein(s)
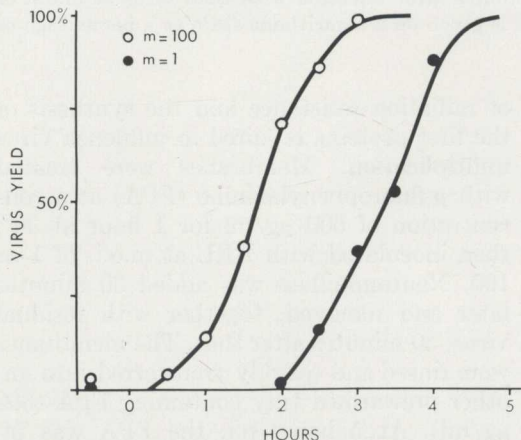

FIG. 4. Development of resistance to ultraviolet light by influenza virus-infected cells. Membranes infected with BEL at m.o.i. of 100 or 1 were irradiated for 40 seconds at the times p.i. indicated on the abscissa. The yield of virus 8 hours p.i. is given as a percentage of that from unirradiated controls.

shortly after infection, whereas low concentrations of the same drug (50–100 μg/ml) affect only the synthesis of viral structural proteins occurring some 1½ hours later. Moreover, when viral multiplication is inhibited by a low concentration (100 μg/ml) of FPA for 5 hours, then allowed to continue by removal of the drug, there is no longer any asynchrony in the release of virus from cells infected at low m.o.i., indicating that multiplicity-dependent delay is over before the synthesis of viral structural proteins begins (White et al., 1965). The same approach was now employed to determine whether delay occurs before or after the inhibition of "early" protein synthesis by higher concentrations of FPA.

Membranes were treated for 1 hour with FPA at a concentration of 100 or 600 μg/ml then inoculated with BEL at various m.o.i. Virus that had not yet penetrated the cells was removed by treatment with neuraminidase half an hour later, followed by rinsing half an hour after that. FPA was immediately replaced and incubation continued until 5 hours p.i. The membranes were then thoroughly rinsed, phenylalanine (100 μg/ml) was added, and incubation continued. At hourly intervals thereafter all the membranes were transferred to a fresh warm tray and the remaining supernatant fluids titrated for their content of virus. The resulting differential yields of virus are plotted in Fig. 6 alongside those from a control set of membranes never subjected to FPA, which were assayed from 3 hours onwards.

The control curves show the shift to the right with decreasing m.o.i. that characterizes multiplicity-dependent delay (Cairns, 1957; White et al., 1965). Membranes treated with the low concentration of FPA (100 μg/ml) begin to release detectable amounts of virus 1¾–2 hours after removal of the drug (or 3 hours after removal in the case of m.o.i. 0.6, where not all cells are infected hence the total yield of virus is lower and that fraction produced before 8 hours falls below the limit of sensitivity of the assay). The differences evident in the control curves between the times of maximum virus production following infection at various m.o.i., represented by the peaks of the differential
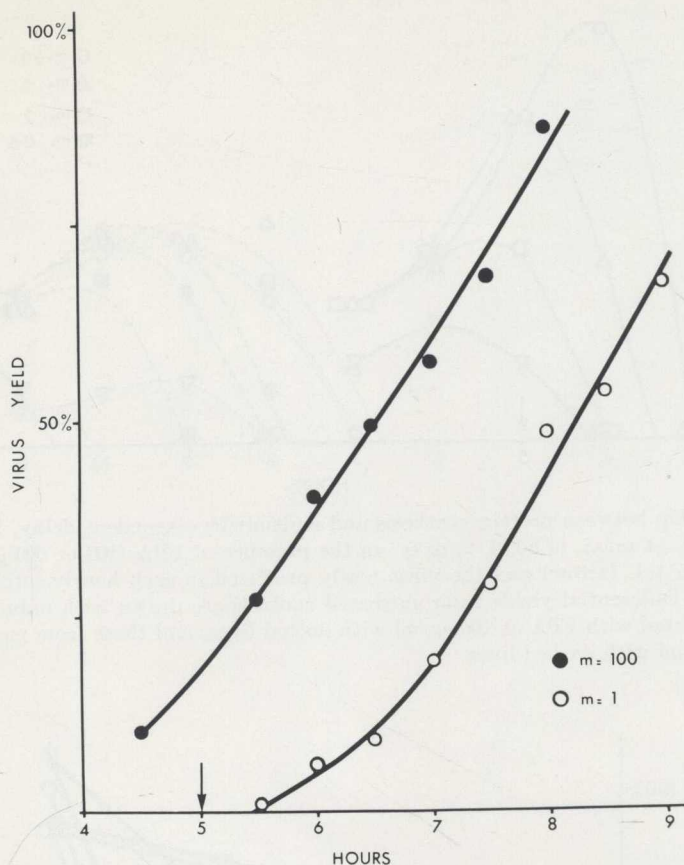
Fig. 5. Relationship between protein synthesis and development of resistance to ultraviolet light. Membranes were inoculated with BEL at m.o.i. of 1 or 100 in the presence of FPA (600 μg/ml). The drug was removed 5 hours p.i. Individual groups of membranes were irradiated for 40 seconds at one of several times p.i. indicated on the abscissa, then reincubated. The yield of virus 11 hours p.i. is given as a percentage of that from unirradiated controls receiving the same treatment.

yields, have disappeared, indicating that the multiplicity-dependent delay has been overcome. Following inhibition by the *high* concentration (600 μg/ml) of the same drug, virus release does not begin until 3 hours after removal of the drug. These curves, by contrast with the other set, do show the progressive shift to the right evident in the controls, indicating that here delay has not been overcome. It may be concluded that multiplicity-dependent delay falls between the points of action of high and low concentrations of FPA, i.e., before the synthesis of viral structural proteins but after that of "early" protein(s).

Delay in the Establishment of Interference

In an attempt to identify more precisely the stage at which multiplicity-dependent delay occurs, an experiment was designed to determine whether it falls before or after the establishment of interference. The interfering virus, BEL, was added at a range of m.o.i. to prewarmed trays of membranes at various times before and after the addition of the challenge virus, SW, at m.o.i. of 50. In each case unadsorbed BEL was rinsed out 30 minutes after its addition, and unadsorbed SW was removed following the addition of neuraminidase 30 minutes after the SW. Eight hours after inoculation with SW,
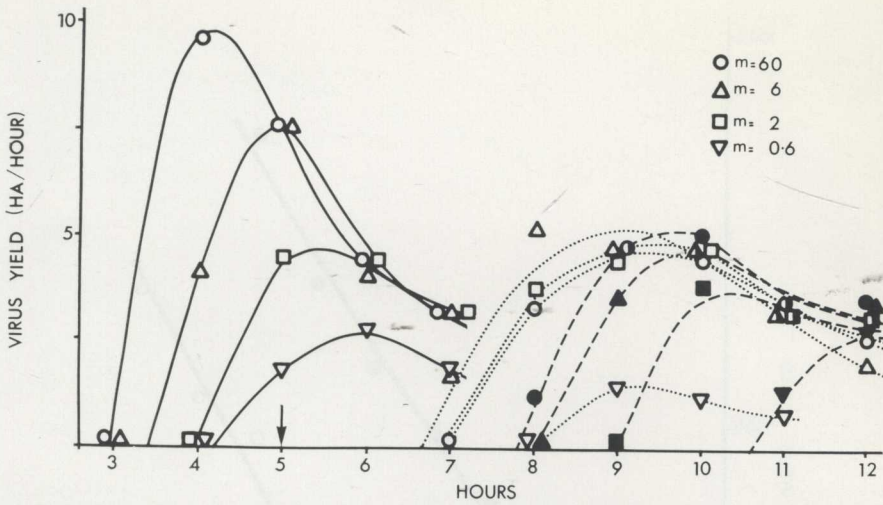
FIG. 6. Relationship between protein synthesis and multiplicity-dependent delay. Membranes were inoculated with BEL at m.o.i. of 60, 6, 2, or 0.6 in the presence of FPA (100 or 600 μg/ml). The drug was removed 5 hours p.i. (arrow) and the virus newly produced in each hourly interval (differential yield) was titrated. Differential yields from untreated controls are drawn with unbroken lines, those from membranes treated with FPA at 100 μg/ml with dotted lines, and those from membranes treated with FPA at 600 μg/ml with dashed lines.
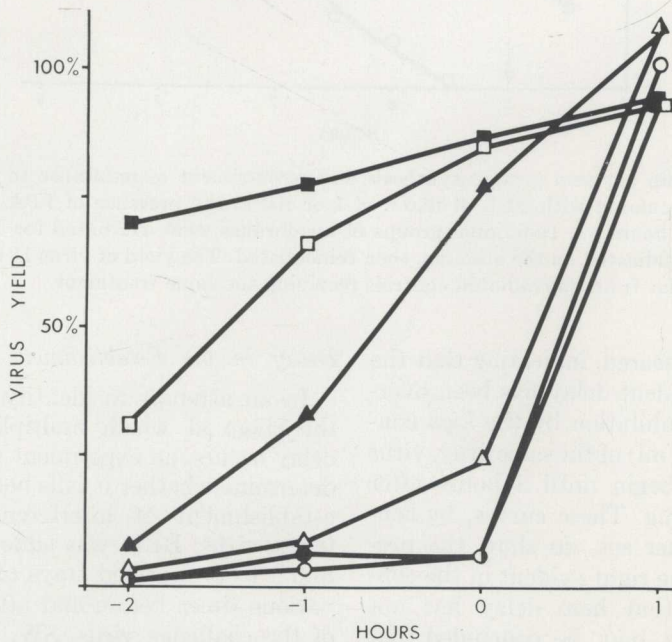

FIG. 7. Delay in the establishment of interference. Membranes were inoculated with BEL at m.o.i. of 200 (open circles), 60 (filled circles), 20 (open triangles), 6 (filled triangles), 2 (open squares), or 0.6 (filled squares), at 2 hours before, 1 hour before, simultaneously with, or 1 hour after inoculation with SW_i at m.o.i. of 50. The yield of SW 8 hours p.i. is given as a percentage of that from controls receiving no BEL.

total yields of that virus were titrated in the presence of a concentration of BEL antiserum demonstrated to inhibit hemagglutination by any of the BEL yield without reducing that attributable to SW. Yields of SW, the challenge virus, are plotted in Fig. 7 as a percentage of the yield from controls receiving no BEL.

At all m.o.i. shown, BEL interferes with the multiplication of SW. At m.o.i. of 6, 20, 60 and 200 BEL administered 2 hours before the challenge dose of SW suppresses the growth of that virus. When given simultaneously with the SW, BEL at m.o.i. of 60 and 200 still inhibits the multiplication of challenge virus almost totally, but BEL at m.o.i. of 6 and 20 interfere less completely. In other words, BEL establishes interference very rapidly at high m.o.i., more gradually at low m.o.i. This leads to the conclusion that influenza virus undergoes a multiplicity-dependent delay before establishing interference. It is not known whether this delay is identical with that characterizing the uncomplicated replication cycle.

DISCUSSION

Irradiation with UVL yields results that are much less equivocal than those based on findings with actinomycin D. In particular, irradiation of cells before infection can hardly affect viral RNA. Barry (1964a,b) has demonstrated that the capacity of chick embryo fibroblasts to support the growth of fowl plague virus is much more sensitive to UVL than their capacity to support the growth of NDV, and the same conclusion has been reached in this paper using a somewhat different virus-cell system. However, when the situation was explored more deeply by carrying out a classical Luria-Latarjet (1947) experiment, it was discovered that the capacity of preirradiated cells to support the growth of BEL is precisely as sensitive as the virus-cell complex itself shortly after infection. In other words, there is some UVL-sensitive cell function, which being more sensitive than, or as sensitive as, the viral RNA itself, is the limiting factor in determining the UVL sensitivity of the cell-virus complex half an hour p.i. Between $\frac{1}{2}$ and

$2\frac{1}{2}$ hours p.i. the complex acquires resistance to radiation, indicating that the need for this UVL-sensitive cell function has disappeared by this time.

It is not easy to equate the acquisition of resistance to UVL with any particular biochemical event. As has been found with poliovirus (Fenwick and Pelling, 1963; Tershak, 1964; Eggers et al., 1965) and herpes simplex (Roane and Roizman, 1964), resistance of the influenza virus-cell complex to UVL will not develop in the absence of prior protein synthesis. Of course this does not imply that the specific biochemical event conferring resistance itself involves protein synthesis, but merely that one or more proteins must be synthesized at some earlier stage. What can be said is that the development of radioresistance by the Sendai virus-cell complex doubtless coincides either with the replication of viral nucleic acid, as found, for example, with pseudorabies virus by Kaplan (1962), or with some event shortly preceding it, as found, for example, with poliovirus by Fenwick and Pelling (1963). By contrast, the transition to radioresistance undergone by the influenza virus-cell complex may be a two-step process, the first step representing escape from nuclear dependence and the second perhaps analogous to that of Sendai. This may account for the difference of about $\frac{3}{4}$ hour between the times at which the complex achieves the state of indifference to actinomycin D (White et al., 1965, Fig. 9) and indifference to UVL (this paper, Fig. 4) respectively, although some or all of this lag may be attributable to the time required for actinomycin to penetrate the cell and bind to DNA. In any case, it can be concluded that cell-coded information becomes redundant at or before $1\frac{1}{2}$–$2\frac{1}{2}$ hours p.i.

Influenza virus has been shown to undergo delay in the course of establishing interference. The results do not permit any conclusions to be drawn about the mechanism of interference in this system, but it is clear that enzymatic destruction of cell receptors is not responsible, because the degree of interference increases so dramatically with time, despite the fact that the interfering

virus was allowed only 30 minutes' adsorption in each instance. It is interesting to note that, when BEL and SW are inoculated simultaneously at equal m.o.i., the yield consists predominantly of BEL, even though the two strains normally multiply at the same rate. A more dramatic example of the same sort of thing is recorded by Forssman (1963), who found that the LEE strain of influenza virus interferes with PR8 more efficiently than vice versa, though LEE multiplies considerably more slowly. A closer examination of this phenomenon may throw further light on mechanisms of interference.

Multiplicity-dependent delay has now been demonstrated to take place after the synthesis of the first "early" protein, but before the last point of action of actinomycin D, before the acquisition of UVL resistance, and before the establishment of interference. The mean delay experienced by a single infecting virus particle has now been determined in a variety of ways (White et al., 1965, Figs. 3, 4, 7, 9; this paper, Fig. 5) and always gives an answer of about $1\frac{1}{2}$ hours.

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Stimulation of Sendai Virus Multiplication by Puromycin and Actinomycin D

THE rate of virus multiplication may ultimately be limited by the number of unprogrammed ribosomes available to viral nucleic acid. There is presumably only a finite probability in unit time that an infecting viral nucleic acid molecule can successfully compete with a vast excess of cellular messenger RNA molecules for an unoccupied ribosome. Even after attachment has been achieved, the subsequent rate of synthesis of viral constituents may be expected to depend on the availability of free ribosomes. This hypothesis was tested experimentally in cells pretreated with two antibiotics, puromycin and actinomycin D, each capable of increasing the number of unprogrammed ribosomes.

Actinomycin D inhibits the synthesis of messenger RNA by binding to DNA and interfering by steric hinderance with the function of DNA-dependent RNA polymerase[1,2]. This leads to the gradual disappearance of polyribosomes with a consequent increase in the number of unprogrammed ribosomes[3] as existing messenger RNA is slowly destroyed during the ensuing few hours[1,3,4]. Sendai is one of several RNA viruses capable of normal multiplication in the presence of actinomycin D[1,5]. It commends itself for the proposed experiment because, by
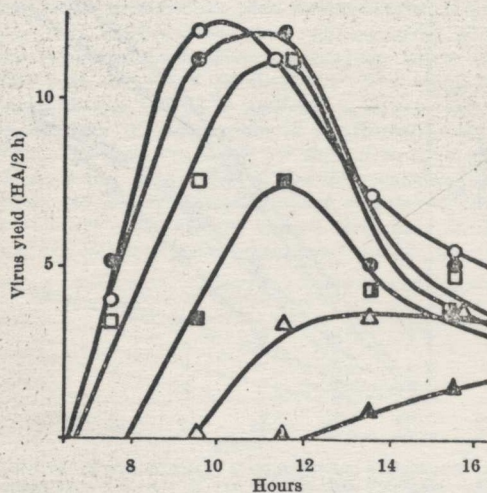

Fig. 1. 'Delay'[10] in the multiplication of Sendai virus. AOS was inoculated with Sendai at m.o.i. of 100 (open circles), 30 (closed circles), 10 (open squares), 3 (closed squares), 1 (open triangles) or 0·3 (closed triangles). The graph shows the amount of haemagglutinin newly produced in each 2-h interval

analogy with other myxoviruses[6,7], it probably does not bring about the early inhibition of host cell protein or messenger RNA synthesis so evident, for example, with mengovirus[8] and poliovirus[4].

The parameters of Sendai virus multiplication were examined in suspended allantois-on-shell[9]. Dilutions of virus were added to pieces of allantois-on-shell (AOS) cut from a single egg and suspended in a simple maintenance medium in a plastic tray. The tray was shaken at 36·5° for 30 min, then 100 units of receptor-destroying enzyme (RDE) added to remove residual inoculum and to restrict virus multiplication to a single cycle. After rinsing, the tissues were transferred to a fresh tray every 2 h and the remaining supernatant fluids titrated for their content of haemagglutinin (Fig. 1).

At high multiplicity of infection (m.o.i.) virus begins to appear after an eclipse period of $6\frac{1}{2}$–7 h and the maximum rate of virus production (given by the peak of the differential curve) is seen during the tenth or eleventh hour post-infection (p.i.). At low m.o.i., on the other hand, the eclipse period is longer and the rate of virus production is still increasing at 16 h. This clearly establishes the occurrence of multiplicity-dependent 'delay'[10] in the multiplication of Sendai virus.

Actinomycin D (0·03, 0·1, 0·3, 1, 3, 10 μg/ml. or nil) was added to AOS 2 h before Sendai virus at various m.o.i., and cumulative yields of haemagglutinin were titrated.
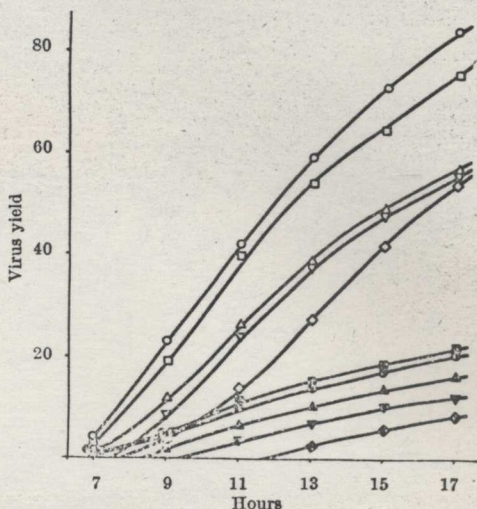

Fig. 2. Stimulation of Sendai virus multiplication by actinomycin D. AOS was inoculated with actinomycin D, then 2 h later with various m.o.i. of Sendai virus. Cumulative yields of haemagglutinin were titrated at 2-h intervals. Closed symbols, controls; open symbols, actinomycin-treated. Circles, m.o.i. 60; squares, m.o.i. 20; triangles, m.o.i. 6; inverted triangles, m.o.i. 2; diamonds, m.o.i. 0·6.

Regardless of m.o.i. and at concentrations of actinomycin of 0.3 μg/ml. to 10 μg/ml. inclusive, the antibiotic markedly increased the rate of virus production. Fig. 2 shows the results of a typical experiment using actinomycin at 2 μg/ml. There is good reason to suppose that the increase would be even greater if the cells could be maintained in actinomycin for a longer period before infection to allow more adequate time for decay of existing messenger RNA.

Puromycin inhibits protein synthesis by removing the developing peptide chains from ribosomes[11]. As a result messenger RNA is released and polyribosomes are disaggregated[12]. AOS was treated with puromycin (1, 3, 10, 30, 100, 300 μg/ml. or nil) for 2 h before infection with Sendai virus (m.o.i. = 1). The drug was removed and the membranes were rinsed immediately before infection, then again on removal of RDE and residual inoculum 30 min later. Cumulative yields of virus were titrated as before. To serve as controls, comparable membranes were treated with p-fluorophenylalanine (600 μg/ml.), which inhibits protein synthesis just as effectively but in a completely different way[13]. At a concentration of 300 μg/ml. puromycin actually retarded multiplication because it could not be removed completely by rinsing. However at concentrations of 30 μg/ml. and 100 μg/ml., puromycin consistently stimulated the rate of virus production. Results from two such experiments are given in Table 1.

Doubtless the degree of stimulation would have been greater had it been possible to remove the puromycin more effectively. That residual traces of the drug actually inhibit virus multiplication can be seen from the fact that pretreatment with puromycin plus actinomycin D gives substantially less stimulation than actinomycin alone. Nevertheless pretreatment with puromycin alone does enhance the rate of virus production. The fact that p-fluorophenylalanine (FPA) is ineffective shows that the stimulating activity of puromycin is attributable not to the inhibition of protein synthesis per se, but to some more specific action of the drug. Had it proved experimentally practicable to use larger concentrations of puromycin or to leave it in until immediately before viral protein synthesis began, the degree of stimulation by puromycin may

Table 1. EFFECT OF PUROMYCIN, ACTINOMYCIN D AND FPA ON THE MULTIPLICATION OF SENDAI VIRUS

Treatment	Cumulative yield of virus (haemagglutinin)				
	9 h	11 h	13 h	15 h	17 h
Experiment 1 (m.o.i. = 0·5)					
Control	–	–	3·0	6·2	9·4
FPA (600 μg/ml.)	–	–	2·3	6·5	9·8
Puromycin (30 μg/ml.)	–	1·2	6·0	12·4	17·6
Actinomycin D (2 μg/ml.)	–	3·8	15·0	29·4	37·4
Actinomycin D + puromycin	–	3·4	13·0	25·0	31·8
Experiment 2 (m.o.i. = 1)					
Control	–	2·8	6·8	10·0	13·2
FPA (600 μg/ml.)	–	1·8	5·8	9·0	12·7
Puromycin (30 μg/ml.)	–	3·4	9·4	15·0	18·6
Actinomycin D (2 μg/ml.)	2·4	15·2	29·6	43·2	53·6
Actinomycin D + puromycin	0·8	7·2	16·0	23·6	30·0

well have been comparable with that obtained with actinomycin.

A recent report by Heller[14] indicates that actinomycin can increase yields of Chikungunya virus by inhibiting the synthesis of interferon and so preventing the late decline in virus production that otherwise occurs following the first cycle of multiplication in cell cultures inoculated with virus at low m.o.i. It seems unlikely that our own results have anything to do with interferon, because all experiments were confined to a single cycle of infection, and stimulation of the rate of virus production is apparent from the moment new virus becomes detectable at the end of the eclipse period. Bukrinskaya and Zhdanov[5] have recently reported a shortening of the Sendai eclipse period by actinomycin D without any increase in viral multiplication rate or total yield. By contrast, Fig. 1 in a paper by Wheelock[15] reveals a slight (25–30 per cent) stimulation of final yield of NDV in the presence of actinomycin without any increase in multiplication rate or shortening of the eclipse period.

Our experiments demonstrate that both actinomycin D and puromycin stimulate the rate of virus production. As a result the yield is also increased (the relative increase being greater the later the yield is titrated) and the eclipse period appears to be shortened because the yield reaches the lower limit of detection earlier. Both antibiotics may act by making available to viral RNA greater numbers of unprogrammed ribosomes.

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4

Brief Communication:

GROWTH OF PARAMYXOVIRUSES IN ANUCLEATE CELLS

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Summary. Anucleate fragments of HeLa cell cytoplasm support the growth of the para-
myxoviruses, parainfluenza type 1 and Newcastle disease virus at least to the point of
development of haemadsorption. These viruses are therefore genetically self-sufficient, insofar
as they can code for all the steps in the multiplication cycle up to this stage without the
participation of nuclear deoxyribonucleic acid (DNA).

INTRODUCTION.

Fundamental differences separate the influenza viruses from the parainfluenza-mumps-measles-
respiratory syncytial-Newcastle disease virus (NDV) group (Waterson, 1962). Indeed, the International
Committee for the Nomenclature of Viruses, as well as most other authorities on classification en-
visage two distinct groups known as myxoviruses and paramyxoviruses respectively (Fenner, 1968). One
of the basic differences is that the multiplication of paramyxoviruses is unaffected or even stimulated
by actinomycin D or by pre-treatment of the cells with ultraviolet light (Barry, Ives and Cruickshank,
1962; Barry, 1964, 1965; Rott and Scholtissek, 1964; Rott, Saber and Scholtissek, 1965; White and
Cheyne, 1965, 1966). This observation has been widely interpreted to mean that the paramyxoviruses
replicate without any requirement for messenger ribonucleic acid (RNA) transcription from the DNA
of the cell nucleus. Definitive proof that paramyxovirus multiplication is totally independent of nuclear
information can come only from a demonstration that such viruses can replicate in anucleate cells.

MATERIALS AND METHODS.

HeLa cells, suspended at a concentration of $10^{6.5}$ - $10^{7.0}$ cells/ml. in Minimal Essential Medium
(M.E.M.) (Eagle, 1959) containing 5% foetal calf serum, were distributed in 3 ml. volumes into
Falcon plastic petri dishes. Each dish was then irradiated with 1400 roentgens from a Stanford model
140-10 X-ray generator with Thermax-T tube operating at 120 kV, 5 mA and 30 cm. Such a dose
prevents further cell division but allows the formation of giant cells which support normal growth of
NDV (Tolmach and Marcus, 1960). The cells were then resuspended, diluted in the same medium to
$10^{5.0}$ - $10^{5.5}$ cells/ml., dispensed into plastic petri dishes and incubated at $37°$ in a humidified atmos-
phere of 5% CO_2 in air. The medium was replenished every 2 days until the 8-10th day when the surviving
cells had achieved diameters up to 20 times those of normal HeLa cells. At this time the cells were
cut by the ingenious method of Marcus and Frieman (1966). A glass-cutting wheel was rolled at
random across the surface of the dish with sufficient pressure to leave a visible line on the plastic
wherever the wheel had passed. About 100 cuts were made in each of three directions, yielding up
to 50 anucleate cell fragments per dish. The dishes were incubated for 30 min. to allow cells to recover
and retract from the cuts.

Virus was then added at a multiplicity of about 10 infectious doses per cell. The following viruses
were used: Parainfluenza type 1 (Sendai)—an egg-adapted strain, passaged repeatedly in the allantois
of chick embryos; obtained originally from the Walter and Eliza Hall Institute. Newcastle disease virus
(NDV), strain NDV/V-8 VRI/66 — an avirulent strain isolated from Australian fowls by the Veterinary
Research Institute, Melbourne; obtained from Dr. E. L. French of the C.S.I.R.O. Division of Animal
Health, and passaged in the allantois of chick embryos. Influenza type A, strain BEL (Burnet and Bull,
1943) — an egg-adapted strain, repeatedly passaged in chick embryo allantois.

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Half an hour after addition of the virus the cells were rinsed to remove unadsorbed virus, or treated for 30 min. with specific antiviral serum for the same purpose (though results were identical with or without antiserum). Fresh M.E.M. - 5% foetal calf serum was added and the dishes were incubated for a further 10-20 hr.

Virus growth was detected by haemadsorption. The cultures were rinsed in ice-cold balanced salts solution, and 5 ml. of an 0·01% suspension of guinea-pig red blood cells in normal saline (RBC) were added. After 40 min. at 4° any RBC not specifically attached to HeLa cells were removed by repeated gentle rinsing. The cells were then fixed in 10% methanol, stained with haematoxylin-eosin, mounted in buffered glycerol and examined by phase contrast microscopy. Staining facilitated the location in cell fragments of any micronuclei which were occasionally observed in these multinucleated cells. Phase contrast microscopy helped to delineate the margins of the cytoplasm, which often stained rather poorly as a result of being spread so thinly.

RESULTS AND DISCUSSION.

Haemadsorbing, anucleate fragments of cytoplasm were frequently and consistently found in cultures infected with Sendai virus in several experiments (Fig. 1). More limited experience with the avirulent V-8 VRI/66 strain of NDV revealed occasional positives. By contrast, in the case of influenza virus, which was included as a control in every experiment (Fig. 2), haemadsorption was found only in cell fragments containing a nucleus.

The findings indicate that the paramyxoviruses, parainfluenza type 1 (Sendai) and NDV/V-8 VRI/66 can initiate infection and lead to the synthesis of viral haemagglutinin in anucleate cell fragments. There was no significant difference, between nucleate and anucleate pieces of cytoplasm, in the time at

Fig. 1. Haemadsorption to anucleate fragments (arrowed) of HeLa cell cytoplasm infected with parainfluenza virus (Sendai). Multiple nuclei are clearly visible in the centre of the giant cell.
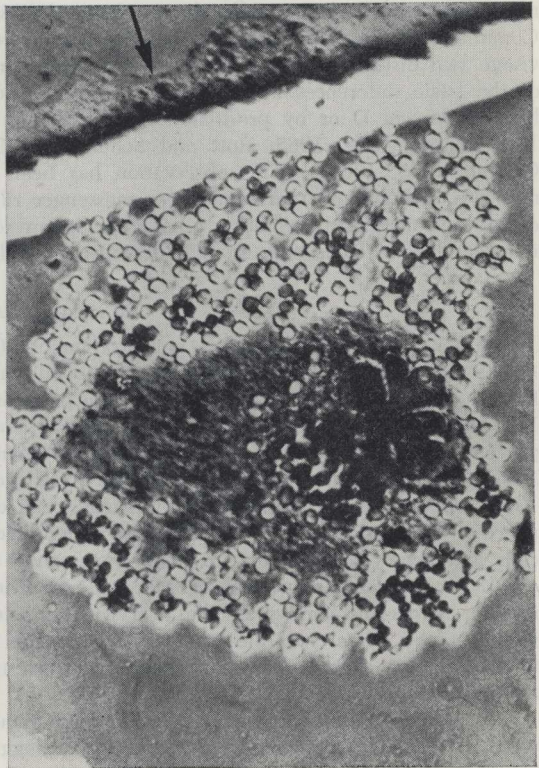
Fig. 2. Absence of haemadsorption to anucleate fragment (arrowed) of HeLa cell cytoplasm infected with influenza virus (Bel). Haemadsorption to the nucleated portion of the cell indicates that the virus will grow in irradiated HeLa cells.

which haemadsorption first became apparent or in the extent of the haemadsorbing area of membrane, indicating that equivalent amounts of haemagglutinin were synthesised in the nucleated and anucleated portions of the cells. Hence, it is highly probable that replication of viral RNA also occurred in the absence of the nucleus. We have no evidence on whether new virions are synthesized or released from such cells, but the results do show that at least the major part of the paramyxovirus multiplication cycle can take place in the absence of a cell nucleus. Marcus and Frieman (1966) confirmed the finding of Crocker, Pfendt and Spendlove (1964) that poliovirus can replicate in enucleated cells, but were unable to find evidence for the multiplication of their virulent strain of NDV.

Anucleate cytoplasmic fragments doubtless contain functional mitochondrial DNA. The role of mitochondrial DNA, RNA and protein synthesis in the normal cell is still far from clear. Nothing at all is known about whether it plays any part in viral multiplication.

The negative results with influenza cannot be interpreted to mean that this virus necessarily requires continuing cellular messenger RNA synthesis for its multiplication. They are to be expected in any case, because influenza RNA and s-antigen are found within the cell nucleus (Liu, 1955; Scholtissek, Rott, Hausen, Hausen and Schäfer, 1962).

There is general agreement that NDV multiplies exclusively in the cytoplasm of normal cells (Wheelock and Tamm, 1959; Wheelock, 1963; Reda, Rott and Schäfer, 1964). The position with Sendai virus is not so clear. Russian workers have presented evidence that Sendai virus RNA and s-antigen synthesis occur in the nucleolus (Zhdanov, Azadova and Kulberg, 1965; and Zhdanov, Azadova and Uryvayev, 1965). The results described in this paper do not rule out the possibility that these viral activities may occur in the nucleolus of the intact cell. However, they do show unequivocally that the cytoplasm of cells lacking a nucleus can indeed support all the stages of the Sendai virus multiplication cycle up to and including the synthesis of viral haemagglutinin.

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The Structural Proteins of Newcastle Disease Virus[1]

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Highly purified Newcastle disease virus has been shown by acrylamide gel electrophoresis to contain three main polypeptides. The probable functional role of each polypeptide has been established by dissociating the radioactively labeled virion with detergents, then separating the resultant subunits by gradient centrifugation, and analysing their composition and function. The nucleocapsid contains a single polypeptide of molecular weight 54,000, which comprises some 45% of the total viral protein. The hemagglutinin and the neuraminidase are composed of polypeptides with molecular weights of 80,000 and 38,000, respectively, which each comprise about 20% of the total viral protein. Other polypeptides make up less than 10% of the total. The purified virion contains little or no host protein.

INTRODUCTION

The paramyxovirus Newcastle disease virus contains approximately 67% by weight of protein, the remaining components being RNA (1%), lipid (24%), and carbohydrate (7%) (Robinson and Duesberg, 1968; Nakajima and Obara, 1967; Blough and Lawson, 1968). Several viral proteins are known. One or more surround the viral RNA forming a helical nucleocapsid which may be isolated intact from virus (Kingsbury and Darlington, 1968; Hosaka and Shimizu, 1968) or from NDV-infected cells (Compans and Choppin, 1967a). The nucleocapsid is encased in a lipoprotein membrane from which project numerous regular spikes (Horne *et al.*, 1960). Associated with the membrane are two activities, the hemagglutinin and the neuraminidase. These are regarded as separate viral proteins (Robinson and Duesberg, 1968). By contrast, the hemolytic activity, adenosine diphosphatase (ADPase), and ad-

enosine triphosphatase (ATPase) which have also been found in preparations of NDV, appear to be proteins of host cell origin (Neurath, 1965; Allison, 1967; Neurath and Sokol, 1963).

This work was undertaken in order to determine the number and identity of the polypeptide monomers forming the NDV proteins. Highly purified NDV has been disrupted with sodium dodecyl sulfate (SDS) and mercaptoethanol, and the resultant polypeptides have been separated by polyacrylamide gel electrophoresis. The hemagglutinin, neuraminidase, and nucleocapsid protein have each been identified with one of the three predominant viral polypeptides.

MATERIALS AND METHODS

Virus. NDV strain V-8 VRI/66 is an avirulent strain isolated at the Veterinary Research Institute, Melbourne, Australia, and kindly supplied by Dr. E. L. French.

Radioisotopes and counting procedures. A mixture of ^{14}C-labeled amino acids purified from *Chlorella* protein hydrolysate was obtained from the Radiochemical Centre, Amersham, England, and stored at −20°. Radioactive samples were counted using an end-window gas-flow Nuclear Chicago counter.

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Gradient centrifugation. All gradients were linear and were preformed by the mixing-chamber method. Sucrose solutions were made up (w/v) in buffered saline (0.85% NaCl in 0.01 M sodium phosphate buffer, pH 7.2) with the addition of 0.001 M disodium ethylenediaminetetracetic acid (EDTA). Potassium tartrate solutions were made up (w/v) in 0.01 M sodium phosphate buffer pH 7.2 with the addition of 0.001 M EDTA. Cesium chloride solutions were made up (w/w) in water.

Hemagglutination assays. Serial 2-fold dilutions of virus in 0.25-ml volumes of physiological saline were made in plastic trays. Fowl erythrocytes were added as an 0.025-ml drop of a 5% suspension. Titers of intact virus were read by the pattern method after 35 min at room temperature. When dissociated virus was assayed, all operations were conducted at 4° to inhibit elution due to neuraminidase activity.

Neuraminidase assays. Samples of 1, 2, or 3 drops were made up to a total volume of 0.2 ml in 0.2 M sodium phosphate-citrate buffer pH 5.5. Two drops (0.05 ml) of fetuin [24 mg protein/ml, prepared by the method of Graham (1961)] were added and the mixture was incubated at 37° for 60 min. Liberated N-acetylneuraminic acid (NANA) was titrated by the 2-thiobarbituric acid method of Warren (1959), except that color was extracted into n-butanol containing 5% 12 N HCl (Aminoff, 1961). Optical density was read at 549 mμ with a Zeiss PMQ II spectrophotometer, and the amount of NANA was calculated from the extinction coefficient of Aminoff (1961). One unit of enzyme is defined as the amount releasing 1 μmole of NANA per minute.

Protein assays. Protein was assayed by the method of Lowry *et al.* (1951) using a standard of bovine serum albumin.

Infectivity assays. Titrations for viral infectivity were carried out in the suspended allantois-on-shell system of Fazekas de St. Groth and White (1958).

Growth and purification of NDV. Fertile hen's eggs were each inoculated with 10^2 to 10^3 egg 50% infectious doses (ID_{50}) of virus, incubated at 36° for 40 hours, then chilled at 4° for 4 hours. The allantoic fluid was clarified by centrifugation for 20 minutes at 1130

g and 4°. Ammonium sulfate was slowly added to a final concentration of 234 g per liter of fluid, and stirring continued for 20 min after the solid had dissolved. The precipitate, deposited by centrifugation at 4° for 20 min at 1130 g, was resuspended in buffered saline to one-tenth the original volume. Insoluble material that did not resuspend was removed by centrifugation. The supernatant was then centrifuged at 25,000 rpm for 40 min in a Spinco 30 rotor to deposit the virus into 0.3 ml cushions of 65% sucrose, placed at the bottom of the tubes. Viral pellets were resuspended in the sucrose cushion together with a little of the supernatant fluid, using a Dounce homogenizer to disperse aggregates of virus. The resuspended virus was clarified at 8500 g for 10 min and repelleted into a sucrose cushion as described. This concentrated virus was layered onto gradients of 15–45% sucrose. It was centrifuged at 30,000 rpm for 15 min in an SW 65 Spinco rotor, or 24,000 rpm for 20 min in an SW 41 rotor, depending on the scale of the preparation. The visible band of virus was collected by Pasteur pipette or by puncturing the bottom of the tube, and dialysed overnight against buffered saline at 4°. The material was then layered onto 20–45% potassium tartrate gradients and centrifuged in an SW 65 or SW 41 Spinco rotor for 3 hours at 50,000 or 40,000 rpm, respectively. The resulting band of pure virus was dialysed at 4° against buffered saline to remove potassium tartrate. It was finally subjected to sonic vibration for 30 sec in an MSE Ultrasonic Power Unit, model 3000, in order to dissociate any aggregates of virus particles. Purified virus was used immediately or stored at −70°.

Growth of radioactively labeled NDV in deembryonated eggs. Fourteen-day-old hen's eggs were deembryonated through the albumin end, leaving the allantoic membrane attached to the shell. This was rinsed twice with standard medium (Fazekas de St. Groth and White, 1958), additionally buffered with 0.005 M Tris pH 7.4. Each egg then received 5 ml of this medium, 0.5 μCi of ^{14}C-labeled amino acids and $10^{7.0-7.5}$ egg ID_{50} of semipurified NDV. The eggs were covered with individual caps of aluminium foil, which were secured to the shell with

paraffin wax, then incubated at 36° on a slowly rotating drum. After 18–24 hours the medium was harvested and the virus purified as described above, omitting the initial ammonium sulfate precipitation.

Preparation of viral polypeptides. Samples were suspended in 2% SDS, 1% 2-mercaptoethanol, and 0.5 M urea in 0.01 M sodium phosphate buffer, pH 7.2, and incubated at 37° for 30 min, then 100° for 1 min (Maizel *et al.*, 1968). They were then dialysed overnight at room temperature against 0.1% SDS, 0.1% 2-mercaptoethanol and 0.5 M urea in 0.01 M sodium phosphate buffer pH 7.2. Dialysed samples were incubated at room temperature for 3 hours in 0.1 M Tris buffer pH 8.0 with iodoacetamide at a final concentration of 0.05 M, and finally redialysed against the dialysis medium described above, the mercaptoethanol being omitted. Specimens were stored at 4° until electrophoresis.

Electrophoresis of polypeptides. A solution containing 5% acrylamide, 0.13% N,N'-methylenebisacrylamide, 0.1 M sodium phosphate buffer pH 7.2, 0.1% SDS, 0.5 M urea and 0.05% N,N,N',N'-tetramethylethylenediamine was polymerised by the addition of 0.1% ammonium persulfate, to form gels of 20 cm length and 6 mm diameter. For optimal separation a sample volume not exceeding 0.25 ml, containing approximately 200 μg protein, or suitable radioactivity, was required. Samples were mixed with 60% sucrose to a final concentration of 10–15%, and overlayered with electrophoresis buffer (0.1% SDS in 0.1 M sodium phosphate buffer, pH 7.2). Electrophoresis was for 16 hours at 6 mA per gel unless otherwise stated. Nonradioactive gels were stained with 0.25% Coomassie brilliant blue in 7% acetic acid for at least 3 hours, and destained by repeated changes of a mixture of 7% acetic acid and 10% ethanol in water. Radioactive gels were fractionated into approximately 70 planchets using the apparatus described by Maizel (1966). The planchets were dried under infrared lamps and counted.

Marker proteins for molecular weight determination. The following proteins were treated with SDS and, where appropriate,

with mercaptoethanol and/or iodoacetamide exactly as described by Shapiro *et al.* (1967): trypsin (once crystallized, Worthington); bovine serum albumin (Armour); cytochrome c (Sigma); human γ-globulin (a gift from Dr. N. Warner, Walter and Eliza Hall Institute, Melbourne). The treated proteins were then used as standards of known molecular weight.

Isolation of nucleocapsid from cells. Chick embryo fibroblast cells were suspended in a spinner culture in Eagle's Minimal Essential Medium (Eagle, 1959) containing one-tenth the usual concentration of amino acids, and supplemented with 5% fetal calf serum. The cells were infected with NDV (30 ID_{50} per cell) in the presence of 30 μCi of ^{14}C-labeled amino acids. Eighteen hours later the radioactively labeled nucleocapsid was extracted according to the method of Compans and Choppin (1967b).

Dissociation of NDV with deoxycholate. To 0.5 ml of a ^{14}C-labeled NDV preparation was added 0.17 ml of 4% sodium deoxycholate in physiological saline buffered with 0.1 M Tris pH 8.0. The mixture was held at 4° for 1 hour with stirring and then fractionated on a sucrose gradient containing 1% sodium deoxycholate.

Dissociation of NDV with a mixture of Tween 80 and ether. To 1.0 ml of ^{14}C-labeled NDV in buffered saline was added 0.025 ml of Tween 80. The mixture was stirred for 30 min at 0°, 0.5 ml of chilled diethylether was added, and the stirring was continued for a further hour. The ether was evaporated in a stream of compressed air, and the sample was dialysed overnight at 4° against buffered saline. The dissociated NDV was then fractionated on a gradient of potassium tartrate. This gradient was used in preference to a sucrose gradient because a reasonable separation of hemagglutinin and neuraminidase was achieved in a relatively short centrifugation time, and because large particles approached density equilibrium near the bottom of the gradient instead of pelleting.

RESULTS

The Purification of NDV

The avirulent V-8 VRI/66 strain of NDV was grown in eggs and purified as described
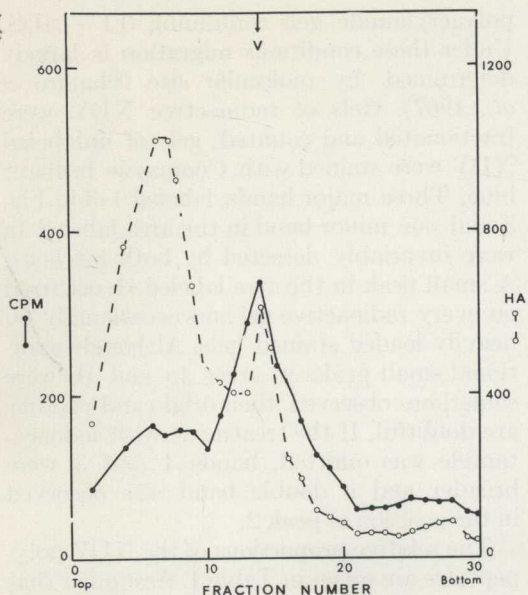
FIG. 1. Purification of NDV by rate zonal centrifugation. Concentrated virus (2 ml) was layered onto a 10-ml gradient of 15–45% sucrose and centrifuged for 20 min at 24,000 rpm in an SW 41 Spinco rotor. Fractions were collected from a puncture in the bottom of the tube and analysed for radioactivity and hemagglutinating activity.

in the Materials and Methods. A 70% recovery of hemagglutinin was obtained by ammonium sulfate precipitation of virus from allantoic fluid. This procedure did not destroy viral infectivity and was considered preferable to adsorption-elution using erythrocytes, because of the considerable hemolysis and variable yields found to be associated with the latter method.

After concentration, the virus was subjected to two cycles of differential centrifugation, and then to a rate-zonal centrifugation on a sucrose gradient. Two peaks of hemagglutinating activity were obtained (Fig. 1). Infectious virus was restricted to the faster sedimenting peak (labeled V in Fig. 1) and to a pellet at the bottom of the tube. The noninfectious material in the more slowly sedimenting peak probably consists of small particles of noninfectious hemagglutinin which are known to be produced in quantity by avirulent strains of NDV (Rott and Schäfer, 1964).

The material from peak V was centrifuged

to equilibrium on a gradient of potassium tartrate. A flocculent narrow band of virus was obtained at a measured density of approximately 1.21–1.22 g/ml (Fig. 2, peak $V1$). Traces of impurities at the top of this gradient cosedimented (in a tartrate gradient) with components of the peak of noninfectious hemagglutinating material. The infectious pellet from the sucrose gradient banded in tartrate at the density of pure NDV, and proved on polyacrylamide gel analysis to contain the viral polypeptides. It was therefore assumed to be clumped virus and was added to the final preparation of pure virus when maximum recovery was required, but it was always excluded from preparations of radioactively labeled virus, in which maximum purity was the objective. Overall recoveries of up to 20% of the initial infectivity and hemagglutinin were obtained. One liter of allantoic fluid yielded 2–5 mg of pure NDV.

The Polypeptide Components of NDV

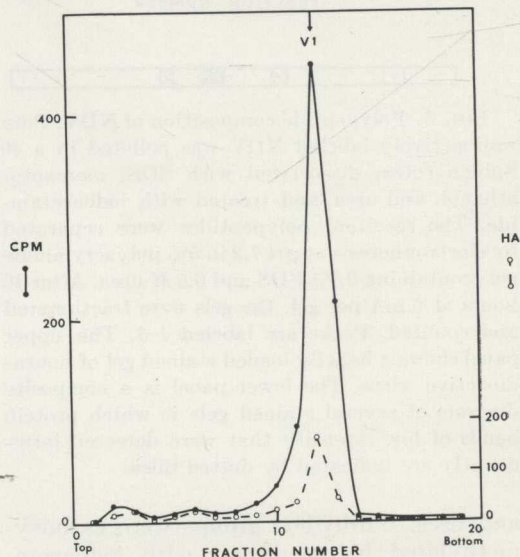Pure NDV was disrupted and reduced using a mixture of SDS, mercaptoethanol,

FIG. 2. Purification of NDV by equilibrium density gradient centrifugation. Fractions 11–19 from the sucrose gradient (Fig. 1) were pooled and dialysed at 4° against buffered saline. A 2-ml sample was then layered onto a 10-ml gradient of 20–45% potassium tartrate. Centrifugation was for 3 hours at 30,000 rpm in an SW 41 Spinco rotor. Fractions were collected and assayed as for Fig. 1.
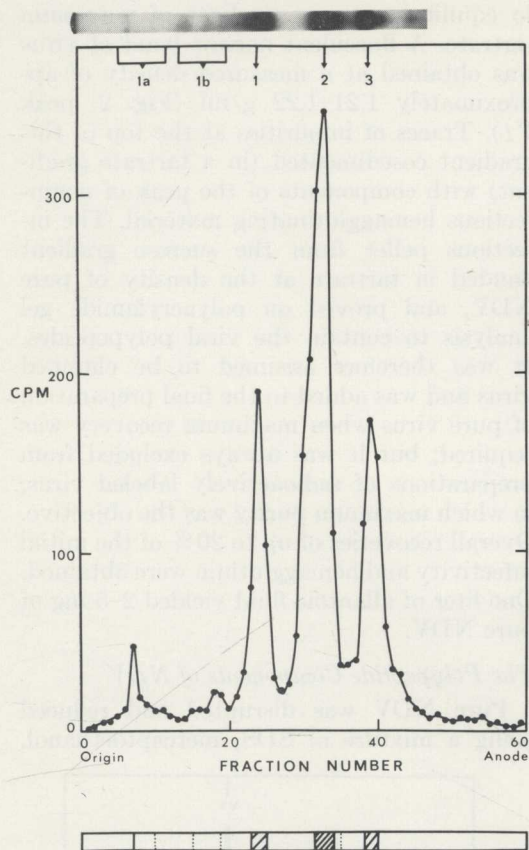
FIG. 3. Polypeptide composition of NDV. Pure radioactively labeled NDV was pelleted in a 40 Spinco rotor, dissociated with SDS, mercapto-ethanol, and urea, and treated with iodoacetamide. The resultant polypeptides were separated by electrophoresis at pH 7.2 in 5% polyacrylamide gels containing 0.1% SDS and 0.5 M urea. After 16 hours at 6 mA per gel, the gels were fractionated and counted. Peaks are labeled 1–3. The upper panel shows a heavily loaded stained gel of nonradioactive virus. The lower panel is a composite diagram of several stained gels in which protein bands of low intensity that were detected infrequently are indicated by dotted lines.

and urea. Sulfhydryl groups were carboxymethylated by treatment with iodoacetamide at pH 8. This digestion procedure was used in order to dissociate the proteins of the intact virus without breaking any covalent bonds other than disulfide bridges. The resultant polypeptides, which retained no hemagglutinating or neuraminidase activity, were separated by electrophoresis on 5%

polyacrylamide gels containing 0.1% SDS. Under these conditions migration is largely determined by molecular size (Shapiro *et al.*, 1967). Gels of radioactive NDV were fractionated and counted; gels of unlabeled NDV were stained with Coomassie brilliant blue. Three major bands, labeled 1–3 in Fig. 3 and one minor band in the area labeled 1a were invariably detected by both methods. A small peak in the area labeled 1b occurred on every radioactive gel and occasionally on heavily loaded stained gels. Although additional small peaks in areas 1a and 1b were sometimes observed, their origin and validity are doubtful. If the treatment with iodoacetamide was omitted, bands 1 and 3 were broader and a double band was observed in the position of peak 2.

The relative proportions of the NDV polypeptides are given in Table 1. Assuming that the ^{14}C-labeled amino acids were incorporated equally into all NDV proteins, peak 2 accounted for 45% of the total viral protein, while peaks 1 and 3 accounted for a further 18% and 21%, respectively. The minor peaks in areas 1a and 1b totalled 9%

TABLE 1

THE RELATIVE PROPORTIONS AND MOLECULAR WEIGHTS OF THE POLYPEPTIDES OF NDV

Electrophoretic peak	Estimated molecular weight[a]	Percentage of virion protein[b]	Molecular ratio per virion[c]
1	80,000	18	1.0
2	54,000	45	3.7
3	38,000	21	2.4
Area 1a	170,000–200,000	5	—
Area 1b	110,000–130,000	4	—
Remainder[d]		7	—

[a] From Fig. 4; the values given are the mean of three separate determinations.

[b] From Fig. 3; the values given are the mean of three separate determinations. The counts in each peak were expressed as a percentage of the total counts recovered from the gels.

[c] Calculated by dividing the percentage of virion protein by the estimated molecular weight and normalizing the resultant ratios with respect to peak 1 as unity.
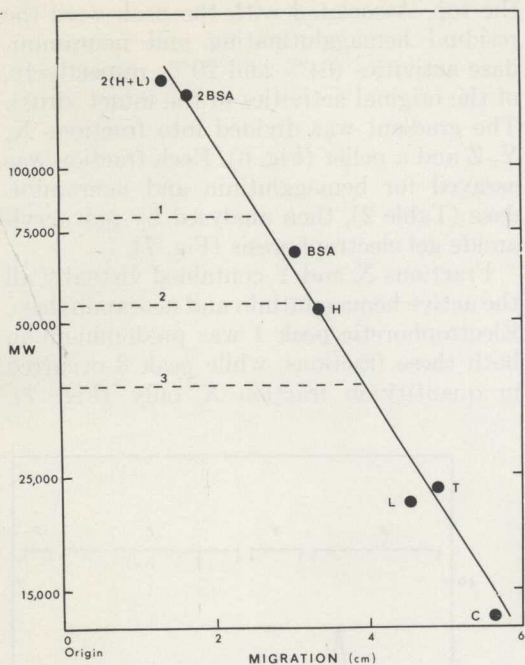
[d] Counts in no particular peak.

FIG. 4. Estimation of molecular weights of NDV polypeptides by electrophoresis. NDV was dissociated with SDS, mercaptoethanol, and urea and carboxymethylated. The polypeptides were separated by electrophoresis for 6.5 hours at 7 mA per gel in parallel with a number of marker proteins of known molecular weight. The gels were stained with Coomassie brilliant blue and the distance migrated by each band was plotted against the logarithm of the known molecular weight of each marker (Shapiro et al., 1967). H or L = heavy or light chain of human γ-globulin; BSA and 2 BSA = bovine serum albumin and its dimer; T = trypsin; C = cytochrome c.

of the viral protein, and the remaining 7% represents low counts smeared along the gel in no particular peak. The values of 80,000, 54,000, and 38,000 for the molecular weights of polypeptides 1, 2, and 3, respectively, were obtained by the method of Shapiro et al. (1967), as shown in Fig. 4. This method is based on the comparative migration in polyacrylamide gels of SDS-saturated viral polypeptides and marker proteins of known molecular weight. The values obtained can only be regarded as approximate.

The high molecular weights of the polypeptides in areas 1a and 1b suggested that these peaks might represent NDV proteins that had been incompletely dissociated, or had subsequently reaggregated. Accordingly, the concentration of urea used during disruption and electrophoresis of NDV was increased from the usual 0.5 M to 8 M, in an attempt to increase the dissociation of the viral polypeptides. However, 8 M urea had no effect on the peak in area 1a (the only high molecular weight peak detected by staining), although of course it reduced the migration of all viral polypeptides.

Identification of the Polypeptides of NDV

In order to correlate each of the polypeptide monomers with known structural or functional components of the virion, a number of different viral subunits were characterized. Thus viral nucleocapsids were isolated from NDV-infected cells, while subunits with hemagglutinating and neuraminidase activities were obtained by dissociating the pure virus with deoxycholate or with Tween 80-ether. The latter two treatments produced less dissociation of viral protein than treatment with SDS, and some biological activity was retained. Functional subunits obtained in these ways were then dissociated to their individual polypeptides by treatment with SDS and analysed by polyacrylamide gel electrophoresis.

Identification of the Nucleocapsid Polypeptide

NDV nucleocapsids are produced in excess during infection and may therefore be isolated from infected cells. Cultured chick embryo fibroblasts were infected with NDV in the presence of ^{14}C-labeled amino acids, and collected 18 hours later. The cells were lysed by osmotic shock, and the nucleocapsids thus released were purified on a CsCl gradient as described by Compans and Choppin (1967b). [The probable fragmentation of the NDV nucleocapsids in the CsCl gradient (Hosaka and Shimizu, 1968) was considered unlikely to affect the identification of the protein monomer.] The nucleocapsids banded at a measured density of 1.3 g/ml, in agreement with the reported values of 1.297 and 1.31 g/ml for the buoyant densities in CsCl of nucleocapsids from the paramyxoviruses SV5 (Compans and Choppin, 1967b) and HVJ (Hosaka, 1968), respectively.
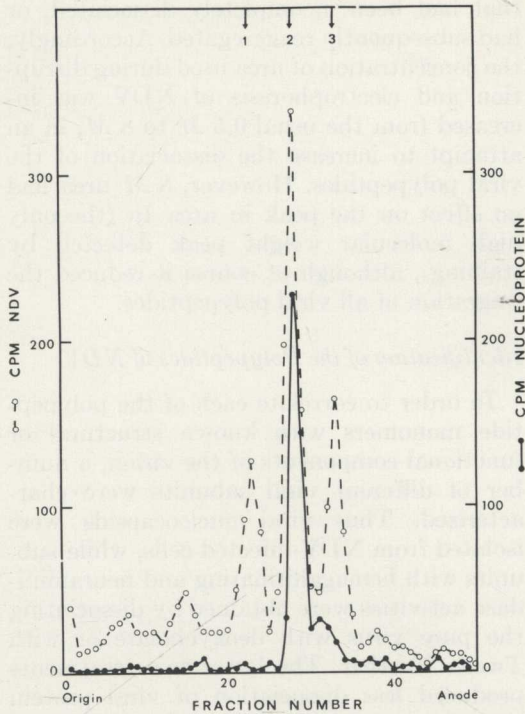
FIG. 5. Identification of the polypeptide of the nucleocapsid protein. NDV nucleocapsids were purified from infected chick embryo cells on a CsCl equilibrium gradient, then dissociated with SDS, mercaptoethanol, and urea, carboxymethylated, and analysed by electrophoresis in parallel with a preparation of pure virus.

Analysis of the nucleocapsid preparation by polyacrylamide gel electrophoresis showed a single polypeptide corresponding to peak 2 of whole virus (Fig. 5). The same result was obtained when nucleocapsids were isolated from allantoic fluid by the method of Rott et al. (1963). Polypeptide 2 was therefore identified as the monomer of the nucleocapsid protein.

Identification of the Hemagglutinin and the Neuraminidase Polypeptides

Dissociation of NDV with deoxycholate. Pure ^{14}C-labeled NDV was disrupted with sodium deoxycholate and the resulting subviral components were partially separated by rate-zonal centrifugation on a sucrose gradient (Fig. 6). Radioactivity was distributed throughout the gradient with a large peak at

the top. Associated with the peak were the residual hemagglutinating and neuraminidase activities (64% and 20%, respectively, of the original activities in the intact virus). The gradient was divided into fractions X, Y, Z and a pellet (Fig. 6). Each fraction was assayed for hemagglutinin and neuraminidase (Table 2), then analysed by polyacrylamide gel electrophoresis (Fig. 7).

Fractions X and Y contained virtually all the active hemagglutinin and neuraminidase. Electrophoretic peak 1 was predominant in both these fractions, while peak 3 occurred in quantity in fraction X only (Fig. 7).
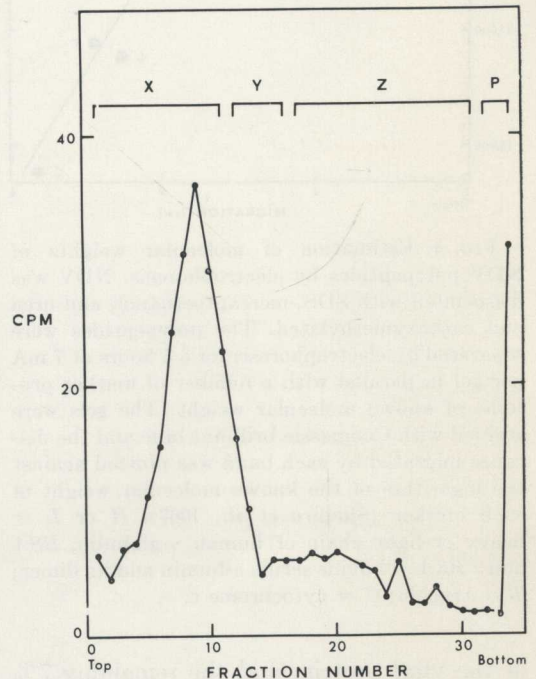

FIG. 6. Rate zonal centrifugation of deoxycholate-dissociated NDV. Dissociated radioactive NDV (0.67 ml) was layered onto a 4 ml gradient of 5–20% sucrose containing 1% sodium deoxycholate and 0.01 M Tris buffer pH 8.0. Centrifugation was for 1 hour at 22,500 rpm and 5° in an SW 65 Spinco rotor. The gradient was collected dropwise from a puncture in the bottom of the tube and analysed for radioactivity. Samples were pooled to give fractions X, Y, and Z. Fraction P was the resuspended pellet. Fractions X-Z were dialysed overnight against buffered saline at 4°, assayed for neuraminidase and hemagglutinating activities (Table 2), and analysed by polyacrylamide gel electrophoresis (Fig. 7).
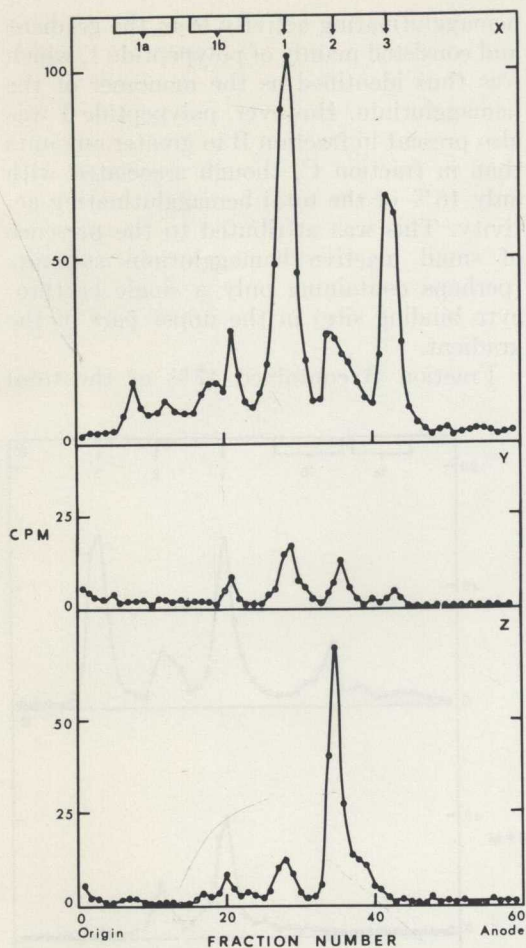
Fig. 7. Polyacrylamide gel electrophoresis of fractions X–Z from the sucrose gradient shown in Fig. 6. Dialysed fractions X–Z were reduced to approximately 0.2 ml by evaporation in a stream of compressed air. Samples were dissociated with SDS, mercaptoethanol, and urea and carboxymethylated before the electrophoresis.

TABLE 2

Biological Activity of Fractions from the Sucrose Gradient Analysis of Deoxycholate-Disrupted NDV

Sucrose gradient fraction[a]	Neuraminidase[b]	Hemagglutinin[b]
X	73	65
Y	18	15
Z	4	<6
P	5	14

[a] From Fig. 6.

[b] Expressed as a percentage of the total activity recovered from the gradient.

These observations suggested that polypeptides 1 and 3 might represent the monomers of the hemagglutinin and neuraminidase, and that the two activities might be separable. When a longer centrifugation time was used the hemagglutinating activity was, in fact, partially separated from the more slowly sedimenting neuraminidase activity. However, better resolution of the two activities was obtained by the method described below.

Polypeptide 2, the monomer of the nucleocapsid protein, was found mainly in sucrose gradient fraction Z and in the pellet P, representing material with a sedimentation coefficient of 300 S or greater.

Dissociation of NDV with a mixture of Tween 80 and ether. In order to identify the individual polypeptides comprising the viral hemagglutinin and neuraminidase, ^{14}C-labeled NDV was dissociated with Tween 80 followed by ether (de-Thé and O'Connor, 1966). This treatment gave greater recoveries of hemagglutinating and neuraminidase activities than the dissociation of NDV with deoxycholate, resulting in a large increase in the hemagglutinin titer and a 40% loss of neuraminidase activity. The subviral components produced were fractionated on a gradient of potassium tartrate (Fig. 8).

Radioactivity was distributed throughout the gradient with a large peak near the bottom. Neuraminidase activity was found near the top of the gradient, partially separated from the hemagglutinating activity, which peaked at an intermediate position. Fractions from these three regions of the gradient, and from the top and bottom, were separately pooled to give five fractions labeled A–E (Fig. 8). Each pool was assayed for hemagglutinin and neuraminidase and then analysed by polyacrylamide gel electrophoresis (Fig. 9).

The polypeptide content of the various subviral components in fractions B, C, and D (Fig. 9) may now be correlated with their biological activities (Fig. 8).

Fraction D contained much radioactivity but relatively little hemagglutinating or neuraminidase activity, and was enriched in the nucleocapsid polypeptide, 2. The predominant subviral component in this part
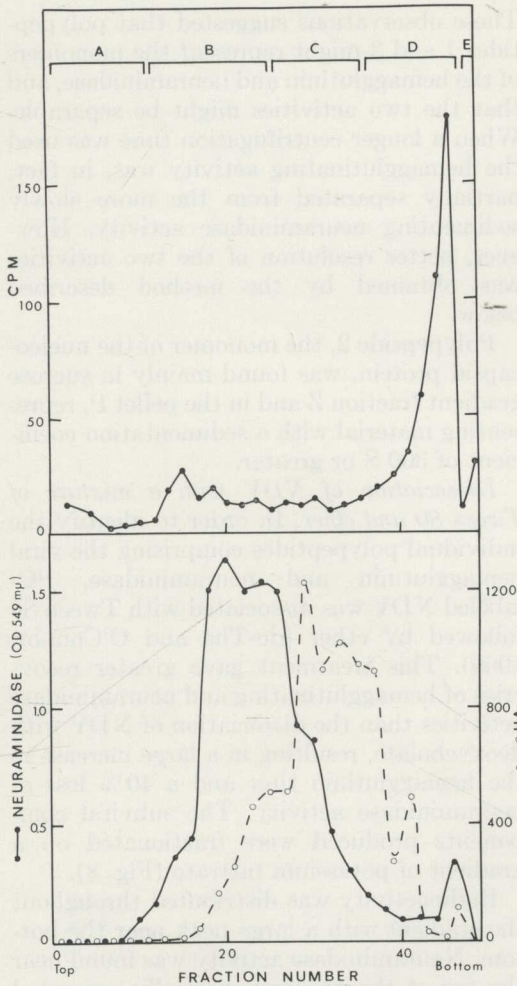
FIG. 8. Gradient centrifugation of NDV after dissociation with Tween 80 and ether. Dissociated NDV (1 ml) was layered onto a 4-ml gradient of 20–45% potassium tartrate and centrifuged for 3 hours at 50,000 rpm in an SW 65 Spinco rotor. Dissociated ^{14}C-labeled NDV was similarly treated. Fractions were collected dropwise from both gradients. The fractions from the nonradioactive gradient were dialysed at 4° overnight against buffered saline and analysed for neuraminidase and hemagglutinating activities. The fractions from the radioactive gradient were analysed for radioactivity, then pooled to give samples A–E. Samples A–E were dialysed, assayed for hemagglutinating and neuraminidase activities, and analysed by polyacrylamide gel electrophoresis (Fig. 9).

of the gradient was taken to be a nucleocapsid core with small amounts of surface protein still attached.

Fraction C contained 58% of the total

hemagglutinating activity from the gradient and consisted mainly of polypeptide 1, which was thus identified as the monomer of the hemagglutinin. However, polypeptide 1 was also present in fraction B in greater amounts than in fraction C, though associated with only 16% of the total hemagglutinating activity. This was attributed to the presence of small inactive hemagglutinin subunits (perhaps containing only a single erythrocyte binding site) in the upper part of the gradient.

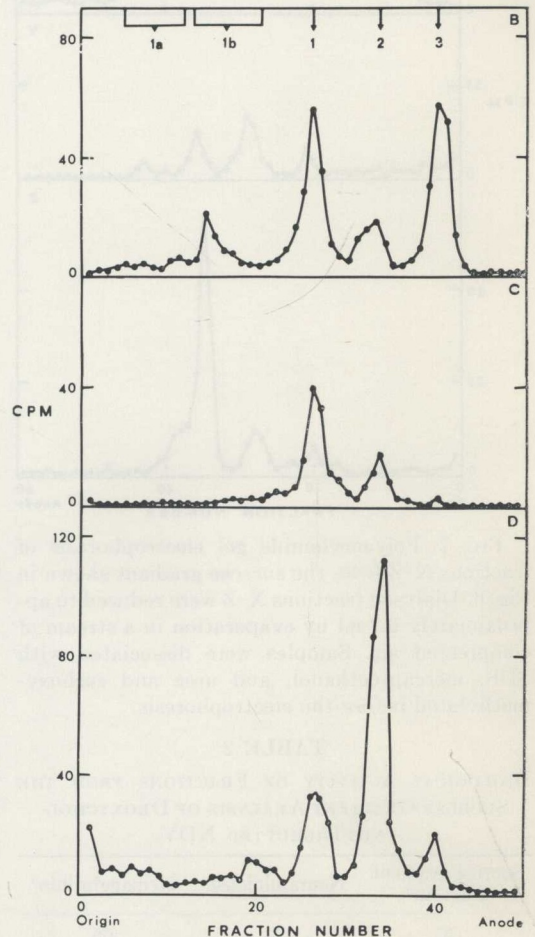Fraction B contained 47% of the total

FIG. 9. Polyacrylamide gel electrophoresis of fractions B–D from the tartrate gradient shown in Fig. 8. Dialysed fractions B–D were reduced to approximately 0.2 ml by evaporation. Samples were then dissociated and subjected to electrophoresis. Fractions A and E contained little biological activity or radioactivity and are not shown.

neuraminidase activity on the gradient and was rich in polypeptides 3 and 1b in addition to polypeptide 1. It was therefore considered that the neuraminidase must be associated with the remaining unidentified polypeptide, 3, perhaps in association with 1b.

In summary, this experiment confirms that the monomer of the nucleocapsid protein is polypeptide 2, and indicates that the monomers of the hemagglutinin and neuraminidase are polypeptides 1 and 3, respectively. In retrospect the same conclusions may be drawn from the analysis of deoxycholate-disrupted NDV (Figs. 6 and 7 and Table 2).

DISCUSSION

Extensive purification of the virus was vital to the subsequent identification of the viral proteins. The purification described here was satisfactory by a number of criteria. It yielded infectious NDV as a visible band of material, which coincided with maximum hemagglutinating activity and maximum radioactivity, at a density of 1.21–1.22 g/ml. Equally critical were the methods used to dissociate and analyze the viral proteins. The advantages of the dissociation of viruses with hot neutral SDS and mercaptoethanol, and the analysis on SDS-containing polyacrylamide gels have been discussed by Maizel et al. (1968). With the addition of a procedure to protect sulfhydryl groups, the method proved eminently suitable for NDV.

NDV was found to contain three main polypeptides (electrophoretic peaks 1, 2, and 3) which had approximate molecular weights of 80,000, 54,000, and 38,000, respectively. Olds and Kingsbury (1968) have reported similar results in a preliminary abstract. As the polyacrylamide gel technique discriminates mainly according to size, it is conceivable that a given peak may contain more than one component of similar size. The three polypeptides accounted for at least 84% of the total viral protein. Since each polypeptide could be identified with a different viral function, none of the main electrophoretic peaks is either a contaminant or an artifact. The status of the high molecular weight peaks in areas 1a and 1b is less definite. Protein polymers are frequently detected at the top of polyacrylamide gels

(e.g., Shapiro et al., 1967; Maizel et al., 1968), and, as viral proteins are naturally prone to aggregate, peaks 1a and 1b may represent aggregates of any of the three main polypeptides or other artifacts of the electrophoresis system. Alternatively, these minor peaks could represent traces of contaminating host proteins, or genuine viral proteins. Since the total counts involved comprise less than 10% of the viral protein, the eventual explanation is unlikely to affect any conclusions regarding the major polypeptide components.

The exact correspondence of the NDV peaks in polyacrylamide gels of radioactive and nonradioactive virus suggests that no major viral polypeptide originates from host cell material existing prior to viral infection. Therefore, if the hemolysin, ADPase and ATPase of NDV are host cell proteins, the quantities incorporated into virus were too small to be detected by the staining technique used.

The identification of a polypeptide (or polypeptides) as the monomer(s) of a viral protein requires, ideally, that the appropriate viral subunit be isolated and purified. The viral nucleocapsid was satisfactorily purified from infected cells, and was also isolated from pure virus. It is undoubtedly composed of polypeptide 2, and probably nothing else. The hemagglutinin and neuraminidase were partially separated by gradient centrifugation. Further purification was not attempted because of the low radioactivity recovered in the active fractions. Both detergent treatments of NDV produced a hemagglutinating subunit (or subunits) which sedimented faster than a second fraction containing much of the neuraminidase activity. Fractions containing the maximum hemagglutinating activity yielded polypeptide 1 as the major component. This strongly suggests that polypeptide 1 is the monomer of the hemagglutinin. Olds and Kingsbury (1968) found this polypeptide to be the major component of a fraction from ether-disrupted NDV that adsorbed to, and eluted from, fowl erythrocytes.

Polypeptide 3 occurred only in fractions containing considerable neuraminidase activity, and is therefore considered to be the monomer of the neuraminidase. However, such fractions were also enriched with a peak

(or peaks) in area 1b, the nature of which is not yet clear. As this peak constitutes only 4 % of the total viral protein, the possibility that it is the neuraminidase, and that peak 3 is therefore a protein of unknown function, appears unlikely, but cannot be completely excluded. Peak 1b may be an inadequately dissociated polymer of polypeptide 3. Alternatively, it may represent a unique polypeptide involved with polypeptide 3 in the structure of the neuraminidase. However, the molecular ratio of polypeptides 3 to 1b in the virion is at least 15:1. A neuraminidase consisting of 15 molecules of molecular weight 38,000, and one of molecular weight 110,000, would itself have a molecular weight of 680,000. Such a molecular weight would be substantially higher than the value of 250,000 which may be estimated from the known sedimentation coefficient of 8–10 S for the NDV neuraminidase (Drzeniek *et al.*, 1966). It is therefore probable that the neuraminidases i composed exclusively of polypeptide 3.

ACKNOWLEDGMENTS

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The Polypeptides of Influenza Virus

I. Cytoplasmic Synthesis and Nuclear Accumulation

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Three major structural peptides were found in radioactive influenza virus type A/Bel that had been grown in calf kidney cells, purified, and analyzed by acrylamide gel electrophoresis. The same three peptides were found in infected HeLa cells. All proved to be stable; none was a precursor of another. The site of synthesis and subsequent migration of these peptides was investigated by cell fractionation. Evidence is presented which suggests that viral peptides were synthesized in the cytoplasm, and that two of them subsequently underwent significant accumulation into the nucleus.

INTRODUCTION

The intracellular localization of influenza antigens has been studied in the past by the technique of immunofluorescence (Schäfer, 1959; Hillis *et al.*, 1960; Fraser, 1967; Rott and Scholtissek, 1967). The S-antigen (ribonucleoprotein) has been generally observed to accumulate first in the nucleus, and only later in the cytoplasm. In contrast, the V-antigen (widely assumed to correspond with the hemagglutinin) is detected exclusively in the cytoplasm. These observations have been interpreted by Rott and Scholtissek (1967) as evidence that both the S- and V-peptides may be synthesized in the nucleus, but that the V-peptides do not assume an antigenic configuration until after they have migrated to the cytoplasm. Furthermore, Becht (1969) has presented autoradiographic data suggesting that nuclear protein synthesis may occur in influenza-invected cells.

In this communication an attempt is made to determine the site of synthesis of individual influenza peptides by combining the following approaches: (1) brief radioactive pulse-labeling of virus-infected cells, followed by a prolonged "chase"; (2) fractionation of cells into nucleus and cytoplasm;

(3) separation of viral peptides by acrylamide gel electrophoresis.

The results demonstrate that all three of the major structural peptides of the influenza virion are synthesized in the cytoplasm, and that two of these subsequently migrate to the nucleus.

MATERIALS AND METHODS

Growth and purification of ^{14}C-labeled influenza virus. Confluent monolayer cultures of primary embryonic calf kidney cells (Lehmann-Grube, 1965) were rinsed twice with Hanks' balanced salt solution and replenished with Minimal Essential Medium (MEM) (Eagle, 1959), modified to contain one-tenth the usual amino acid concentrations and supplemented with 0.1% bovine serum albumin. Influenza virus A/Bel (Burnet and Bull, 1943), grown in the allantois of developing chicken embryos, was inoculated into the cultures at a multiplicity of infection (m.o.i.) of 0.05 tissue culture ID_{50} per cell together with ^{14}C-labeled amino acid mixture (Amersham CFB. 104 at 52 mCi/mAtom carbon) to a final concentration of 0.25 μCi/ml. The cultures were incubated at 35° for 72 hours, by which time they showed marked cytopathic effects.

Virus was then purified by the following modification of the procedure developed for Newcastle disease virus (Haslam *et al.*, 1969). The supernatant tissue culture fluid was clarified by centrifugation (12,000 g, 10 min), the virus was deposited (64,000 g, 30 min) onto a small cushion of 2.3 M sucrose, resuspended by brief sonic vibration, and purified by rate zonal centrifugation in a 15–60% (w/v) sucrose gradient (64,000 g, 120 min), followed by equilibrium centrifugation in a 20–40% (w/v) potassium tartrate gradient (174,000 g, 180 min). Fractions were collected dropwise from the bottom of the tube and assayed for hemagglutinin and ^{14}C. Peaks of the two activities coincided and corresponded to the visible band in both gradients.

Infection and pulse-labeling of HeLa cells. Suspension cultures of HeLa S3 cells were propagated in MEM supplemented with 5% fetal calf and 5% bovine serum. For virus infection, cells prelabeled for 48 hours with thymidine-^3H (at 0.07 $\mu Ci/ml$ and 35.7 mCi/mmole) were washed once and then resuspended at a concentration of 10^7 cells/ml in serum-free MEM modified to contain one-tenth the usual amino acid concentrations. Influenza A/Bel propagated in embryonated hen's eggs and concentrated by centrifugation, was added at an input multiplicity of 300 egg ID$_{50}$ per cell, and allowed to adsorb for 30 min at room temperature. The cell suspension was then diluted 10-fold with prewarmed MEM, lacking amino acids and supplemented with 5% dialyzed fetal calf serum. Cultures were incubated at 37° with stirring for a further 3.5 hours. The

cells were then gently centrifuged (250 g, 5 min) and resuspended in amino acid-free medium at a concentration of 4×10^7 cells/ml. After a 10-min recovery period, ^{14}C-labeled amino acid mixture was added to a final concentration of 10 $\mu Ci/ml$. After labeling for 3 min, a chase in the presence of unlabeled amino acids was achieved by diluting the cells 40-fold with complete growth medium. At 1 and 60 min, respectively, aliquots of 2×10^7 cells were diluted into ice-cold phosphate-buffered saline (PBS) (Dulbecco and Vogt, 1954). These chase conditions inhibit further incorporation of ^{14}C-amino acids into acid-insoluble material (Table 1).

Cell fractionation. Cells were washed twice in ice-cold PBS, then swollen for 15 min in 1 ml of hypotonic buffer (0.01 M KCl, 0.002 M MgCl$_2$, and 0.01 M Tris at pH 7.4), and disrupted in a Dounce homogenizer (Penman *et al.*, 1963). After centrifugation (1000 g, 5 min), the pellet comprised predominantly nuclei, but also small numbers of unbroken cells, as well as cell debris. Since it was imperative to obtain an uncontaminated preparation of clean nuclei, even at the expense of some loss of nuclei, the specimen was subjected to further purification as follows. The pellet was resuspended in 0.9 ml of cold TKM buffer (0.025 M KCl, 0.005 M MgCl$_2$, and 0.05 M Tris at pH 7.4) containing 1.5% by volume of the nonionic detergent Nonidet P40 (Shell Chemicals) (O'Brien, 1964), in order to rupture cytoplasmic membranes and remove cytoplasmic material from around unclean nuclei (Borun *et al.*, 1967). The suspension was then mixed

TABLE 1

DEMONSTRATION OF THE VALIDITY OF THE CHASE PROCEDURE[a]

	Duration of cold amino acid chase (min)				
	1	15	30	45	60
TCA-precipitable ^{14}C-amino acid counts	185 (16)	170 (10)	165 (15)	180 (6)	160 (10)

[a] Virus-infected cells were labeled for 3 min with ^{14}C-amino acids and then chased by the addition of unlabeled (cold) amino acids, as described above. At the indicated times, aliquots of cell suspension were chilled and precipitated with an equal volume of 20% trichloroacetic acid. The acid-precipitable counts given in the table are the mean of three determinations, and the figures shown in parentheses represent half the maximum range of those assays.

with 2.1 ml of 2.3 M sucrose in TKM buffer, underlaid with 1 ml of 2.3 M sucrose in TKM and centrifuged at 120,000 g for 30 min. The resulting nuclear pellet and the original intact cells were assayed for radioactivity (^{14}C and ^{3}H) in trichloroacetic acid-precipitable material using an Ansitron scintillation counter. The recovery of nuclei was equated with the recovery of thymidine-^{3}H (usually 20–40 %).

Acrylamide gel electrophoresis. Electrophoresis on 5 % acrylamide gels was performed as described by Maizel (1966) with the following modifications. Samples were dissociated with 2 % sodium dodecyl sulfate, 0.5 M urea, 0.04 M 2-mercaptoethanol, and 0.1 M dithiodiethanol in 0.01 M phosphate buffer pH 7.2 at 37° for 30 min, then at 100° for 1 min, and finally, made 10 % in glacial acetic acid. Mercaptoethanol was omitted from the dialysis buffer.

RESULTS

The Structural Peptides of Influenza Virus

Influenza virus, labeled with ^{14}C-amino acids, was grown in calf kidney cells, purified, and analyzed by gel electrophoresis (Fig. 1a).

The virus possesses three major structural peptides, referred to here as VP1, VP2, and VP3. The identification and characterization of these and minor components of the virion, such as the shoulder sometimes detectable on the anodal side of VP1, is currently under study.

Viral Peptide Synthesis in Infected Cells

Suspension cultures of HeLa cells infected 4 hours earlier with influenza virus were labeled for 15 min with ^{14}C-amino acids then analyzed by gel electrophoresis (Fig. 1b). Fig. 1c shows the electropherogram of mock-infected cells. It is apparent that the infected cell produces predominantly the three main structural peptides of the virion.

Nuclear-Cytoplasmic Fractionation of Infected Cells

In order to determine the site of synthesis of these viral peptides, cells infected 3.8 hours earlier were labeled with ^{14}C-amino
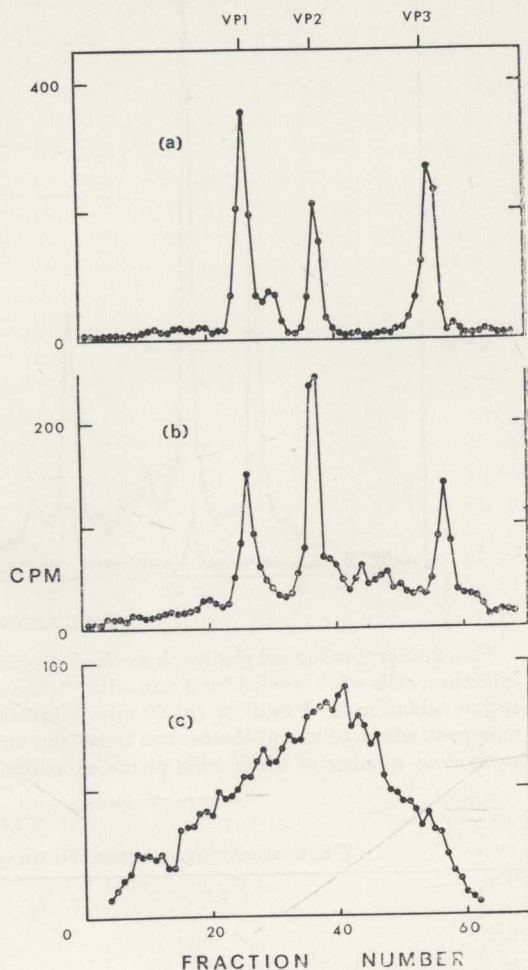

FIG. 1. Acrylamide gel electrophoresis of ^{14}C-amino acid-labeled peptides from (a) purified influenza virus grown in calf kidney cells, (b) HeLa cells infected for 4 hours with influenza virus then labeled for 15 min, (c) mock-infected HeLa cells. Migration is toward the anode on the right.

acids for 3 min, chased with unlabeled amino acids for 1 or 60 min, then separated into nuclear and cytoplasmic fractions and analyzed by gel electrophoresis. A chase of at least 1 min was necessary to ensure the completion of most of the peptides still attached to polyribosomes at the termination of the pulse (Shapiro *et al.*, 1966) In Fig. 2a, the electropherogram of the nuclei is compared with that of the whole cells after the 1-min chase. The three viral peptides can be clearly seen in the whole cell; at the same
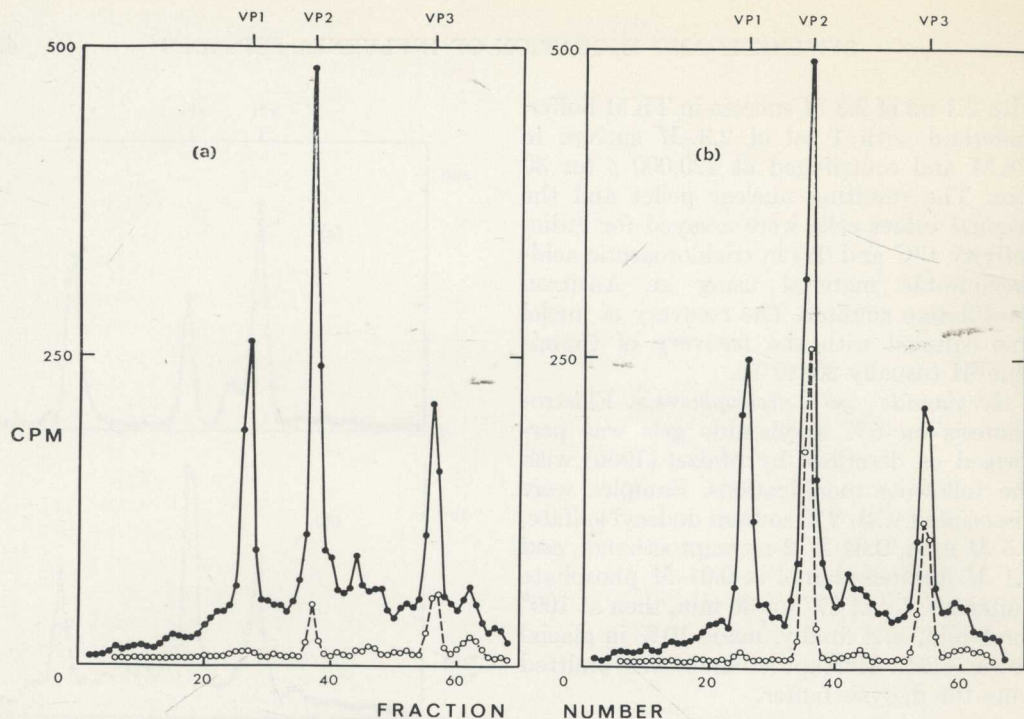
FIG. 2. Acrylamide gel electrophoresis of peptides in influenza virus-infected cells. At 3.8 hours after infection, cells were labeled for 3 min with ^{14}C-amino acids and then chased in the presence of unlabeled amino acids for (a) 1 min, or (b) 60 min. Migration is toward the anode on the right. The solid lines represent whole cells and the broken lines, the nuclei. The data have been normalized to represent an equivalent number of whole cells or nuclei as described in Table 2.

TABLE 2

DETERMINATION OF THE NORMALIZATION FACTOR USED IN FIG. 2[a]

	Duration of cold amino acid chase (min)	
	1	60
^{14}C-amino acid/^{3}H-thymidine ratio per aliquot of:		
Whole cells	0.995	0.984
Nuclei	0.185	0.279
Hence fraction of labeled protein in nucleus of cell =	$\dfrac{0.185}{0.995}$	$\dfrac{0.279}{0.984}$
	= 0.186	= 0.283
Activity (^{14}C cpm) layered in electrophoresis of:		
Whole cells	3996	3250
Nuclei	1944	3654
Hence normalization factor for ordinate scale of electropherograms of nuclear material in Fig. 2 =	$\dfrac{3996}{1944}\cdot 0.186$	$\dfrac{3250}{3654}\cdot 0.283$
	= 0.382	= 0.252

[a] This normalization was applied because of variation in the recovery of nuclei in the extraction procedure and also because of the different amounts of radioactive sample used in each electrophoresis. Note, however, that the use of the above ^{14}C/^{3}H ratios, obtained with an Ansitron scintillation counter, eliminates the need to derive nuclear recoveries in the deduction of the normalization factors. Actually, in the above experiment the nuclear recoveries, as obtained using ^{3}H-thymidine data, were 40.5 and 20.4%, respectively.

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TABLE 3

PERCENTAGE OF EACH VIRAL PEPTIDE FOUND IN THE NUCLEUS[a]

Duration of cold amino acid chase (min)	VP1	VP2	VP3
1	2.2	9.0	36.6
60	2.7	47.0	64.1

[a] Expressed as a percentage of the amount of the corresponding peptide in the whole cells. Data are derived from Fig. 2.

time, the nuclear fraction contains only a minor portion of them.

Figure 2b shows the results obtained when the period of chase in unlabeled amino acids was increased from 1 min to 60 min. The data indicate clearly that all three viral peptides are stable; none is a short-lived precursor of the type encountered in polio-virus-infected cells (Maizel and Summers, 1968). The most striking result, however, is that VP2 and VP3 have been chased into the nuclear fraction. In contrast, VP1 has still not left the cytoplasm.

The data contained in Fig. 2 is expressed quantitatively in Table 3, which gives the percentage of each viral peptide from the whole cell that is present in the nuclear fraction at the two chase times.

DISCUSSION

Acrylamide gel electrophoresis of purified influenza virus type A/Bel has demonstrated three major structural peptides. Duesberg and Robinson (1967) previously reported two or three peptides in purified influenza type A/PR8, analyzed by a somewhat different electrophoretic technique.

The primary purpose of this communication has been to demonstrate certain aspects of the viral infection cycle in a line of HeLa cells conveniently handled in suspension culture. It should be noted that this experimental system is one in which the virus undergoes an abortive cycle of multiplication, not leading to any significant production of infectious virus (Henle et al., 1955).

Furthermore, the high multiplicities of infection necessary for meaningful biochemical studies would result in the production of incomplete virus even in a permissive cell line (von Magnus, 1954). Nevertheless, it is apparent from the experiments reported in this paper that HeLa cells infected at high m.o.i. synthesize three major peptides which comigrate in acrylamide gel electrophoresis with the three major structural peptides of purified virus grown in another cell system, namely calf kidney cells.

The pulse-chase experiment, the results of which are depicted in Fig. 2 and expressed quantitatively in Table 3, demonstrates three main facts. (1) After a 3-min label and a 1-min chase, only a minor portion of each of the three newly synthesized viral peptides is in the nuclear fraction. (2) During the 60-min period of chase, two of the viral peptides, VP2 and VP3, then show significant accumulation into the nuclear fraction. (3) The remaining peptide VP1, does not accumulate in the nuclear fraction. Similar results have been obtained in several comparable experiments.

The obvious and most interesting interpretation of these data is that the three major structural peptides of the virus are synthesized in the cytoplasm, and that eventually two of them, VP2 and VP3, accumulate in significant amounts in the nucleus, but the third, VP1, does not. Before discussing this interpretation further, it is essential to consider possible alternative explanations.

As yet there is no cell fractionation procedure that can guarantee absolute purity as well as integrity of mammalian cell nuclei. Attempts to "clean" isolated nuclei of adherent cytoplasmic contamination may lead to loss of nuclear material in the process. Hence, the possibility cannot be entirely discounted that influenza peptides may be synthesized in the nucleus then leak out of the nucleus during cell fractionation. Following a 60 min chase, VP2 and VP3 may have become incorporated into a structure too large to leak out in this way, e.g. nucleocapsid. Conversely, the presence of VP2 and VP3 in the nuclear fraction is unlikely to be

due to contamination with cytoplasmic material, as VP1 is not found in the nuclear fraction at all, while the content of VP2 and VP3 increases markedly with the time of chase. Nevertheless, the possibility cannot be entirely discounted that VP2 and VP3 become associated in a time-dependent fashion with some structure which cosediments with nuclei.

Perhaps these viral peptides enter the nucleus in the course of simple diffusion around the cell. The smaller the molecule the more rapidly it may be expected to enter the nucleus.

Work is proceeding to complete the identification of these three viral peptides. Preliminary data suggest that VP1 is the peptide of the viral hemagglutinin, and VP2 that of the ribonucleoprotein. The older fluorescent antibody work can now be reinterpreted in the light of the results contained in this paper. The presence of S-antigen in the nucleus and V-antigen in the cytoplasm should not be taken to mean that their constituent peptides are made in those sites. Both the ribonucleoprotein and the hemagglutinin peptides are synthesized in the cytoplasm, then one of them migrates to the nucleus. The identity of the peptide, VP3, perhaps corresponding to the viral neuraminidase, and the reason for its migration to the nucleus now become matters of particular interest.

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The Polypeptides of Influenza Virus

II. Interpretation of Polyacrylamide Gel Electrophoresis Patterns[1]

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The polypeptides of purified influenza virus strain A/Bel have been examined by polyacrylamide gel electrophoresis in the presence of neutral sodium dodecyl sulfate. The virus contains two polypeptides and two glycoproteins, one of the latter being a very minor component. The two polypeptides are both relatively rich in arginine and methionine, and the major glycoprotein is relatively rich in cysteine. This glycoprotein is labile, breaking down or disaggregating under certain conditions to yield two or more derivative electrophoretic peaks. The stability and electrophoretic mobility of this glycoprotein depend upon the host cell in which the virus is grown, suggesting that, while the protein moiety is specified by the virus, the carbohydrate varies with the host cell. The polypeptides of influenza strain B/Lee show many similarities to those of the A/Bel strain.

The polypeptides synthesized in various lines of cultured cells during productive or non-productive infection with Bel virus correspond with those of the purified virion, except for small differences in the electrophoretic mobility of the major glycoprotein.

INTRODUCTION

Proteins comprise approximately 70 % of the mass of the influenza virus particle and occur in at least three biologically distinct forms: the hemagglutinin, the neuraminidase and the internal nucleocapsid (see Robinson and Duesberg, 1968). The virus also contains carbohydrate, some of which is covalently bound to the hemagglutinin (Laver and Webster, 1966; Frisch-Niggemeyer and Hoyle, 1956). In order to determine the number of different polypeptide species in the viral proteins, the virus may be dissociated by sodium dodecyl sulfate (SDS) in combination with reagents that break

disulfide bonds, and analysed by the polyacrylamide gel electrophoretic method of Maizel (1969). Using this technique a number of workers have recently reported somewhat conflicting results. Three (Griffith, 1968; Taylor *et al.*, 1969; Dimmock and Watson, 1969; Holland and Kiehn, 1970) or four (Pons and Hirst, 1969; Joss *et al.*, 1969) major electrophoretic components and several minor species have been observed. Such discrepancies could reflect the use of different host cells, virus strains, dissociation conditions, or electrophoretic techniques.

The present paper represents an attempt to investigate these variables systematically. The polypeptides of influenza strain A/Bel, grown in two host cell systems and dissociated by several methods in common use, have been compared. Several factors have been found to influence the observed polypeptide pattern, and the chief source of variation has been identified as a glycopro-

tein. The components of a second strain of
influenza (B/Lee) have also been investi-
gated. The identification of the constituent
polypeptides of each of the virion proteins
is the subject of an accompanying report
(Haslam et al., 1970).

MATERIALS AND METHODS

Many of the materials and methods used
in this work have already been described in
detail by Haslam et al. (1969) or Taylor et
al. (1969) and will not be repeated here.

Virus. Stocks of influenza strain A/Bel
(Burnet and Bull, 1943) and B/Lee (Francis,
1947) were propagated in fertile hen's eggs.

Radioisotopes and counting procedures. The
following isotopes were obtained from the
Radiochemical Centre, Amersham, England:
^{14}C-labeled amino acid mixture (CFB.104),
D-glucosamine-1-^{14}C hydrochloride (CFA.
422), DL-valine-^3H(G) (TRA.62), L-arginine-
^{14}C(U) monohydrochloride (CFB.8), L-cys-
teine-^{14}C(U) hydrochloride (CFB.108), L-
glutamic acid-^{14}C(U) (CFB.10), L-lysine-^{14}C
(U) monohydrochloride (CFB.15), L-methi-
onine (methyl-^{14}C) (CFA.152). Samples con-
taining ^{14}C only were counted on planchets
using an end-window gas-flow Nuclear
Chicago counter. Samples containing ^3H, or
a mixture of ^{14}C and ^3H were counted in an
Ansitron scintillation counter using a scintil-
lation fluid containing PPO (2,5-diphenyl-
oxazole, 5 g) and dimethyl POPOP (1,4-
bis-[2-(4-methyl-5-phenyloxazolyl)]benzene,
0.1 g) per liter of toluene. To prepare poly-
acrylamide gels for scintillation counting,
the gel fractions were collected in vials and
dried at 160°; 0.5 ml of a mixture of 25%
NCS (Nuclear Chicago solubilizer) and
3.75% water in toluene was then added
(Basch, 1968). The vials were capped and
heated overnight at 37°. Scintillation fluid
(10 ml) was added and the samples were left
overnight at 4° and finally counted.

*Production of radioactive virus in calf kid-
ney cells.* Confluent monolayer cultures of
primary embryonic calf kidney were in-
fected with the Bel or Lee strains of influenza,
in the presence of a ^{14}C-labeled amino acid
mixture as described by Taylor et al. (1969).
The cultures were incubated for 72 hours
and the virus yield purified. Virus containing

^3H-labeled valine was grown in cells sus-
tained by Eagle's Minimal Essential Me-
dium (MEM) (Eagle, 1959) which was
modified to contain one-tenth the normal
valine concentration, 0.1% bovine serum
albumin, and 3-15 μCi/ml ^3H-labeled valine.
This medium was further modified to con-
tain one half the normal glucose concentra-
tion and 0.2 μCi/ml ^{14}C-labeled glucosamine
in order to obtain doubly labeled virus.

Production of radioactive virus in eggs. Ten-
day-old fertile hens' eggs were inoculated
with 10^2-10^3 50% egg infectious doses
(EID_{50}) of virus, and incubated at 36° for
24 hours. A mixture of ^{14}C-labeled amino
acids was then injected (3 μCi per egg), and
the eggs were incubated for a further 24
hours (Bel) or 48 hours (Lee).

*Purification of nonradioactive virus (Bel or
Lee) from allantoic fluid.* The allantoic fluid
was clarified by centrifugation (1000 g, 20
min). Virus was concentrated from the super-
natant by precipitation with ammonium
sulfate (Haslam et al., 1969), or by adsorp-
tion to fowl erythrocytes. The eluate from
the erythrocytes contained 30-50% of the
original hemagglutinating activity, com-
pared with 50-70% recovered by the am-
monium sulfate precipitation method. The
concentrated virus was then deposited
(64,000 g, 40 min) onto 0.5 ml cushions of
65% (w/v) sucrose. The viral "pellets" were
resuspended and further purified by rate
zonal centrifugation in a 15-60% (w/v)
sucrose gradient (64,000 g, 90 min), followed
by equilibrium centrifugation in a 20-40%
(w/v) potassium tartrate gradient (174,000
g, 180 min). One liter of allantoic fluid
yielded approximately 2.0 mg of purified
viral protein.

Purification of radioactively labeled virus.
Tissue culture medium or allantoic fluid was
clarified by centrifugation (12,000 g, 10
min). The virus was then deposited onto a
sucrose cushion and further purified as de-
scribed above. Strain Lee was not purified
beyond the sucrose gradient stage.

*Radioactive labeling of viral polypeptides in
cells.* Confluent monolayer tube cultures of
primary embryonic calf kidney or other cells
were washed twice with MEM from which
all amino acids had been omitted (MEM-

TABLE 1

METHODS OF DISSOCIATING INFLUENZA VIRUS FOR ELECTROPHORESIS

Method	Treatment solution	Incubation conditions	Dialysis medium	Second treatment
1. Taylor *et al.* (1969)	2% SDS, 0.5 M urea, 0.04 M 2-mercaptoethanol, 0.1 M 2-hydroxyethyl disulfide, 0.05 M sodium phosphate buffer pH 7.2	30 min, 37° then 1 min, 100°	0.1% SDS, 0.5 M urea, 0.01 M sodium phosphate buffer, pH 7.2	None
2. Modified from Fine *et al.* (1968)	As method 1, except that sodium phosphate buffer was replaced by 0.05 M sodium bicarbonate buffer pH 10.6	5 min, 80°	As method 1	None
3. Haslam *et al.* (1969)	2% SDS, 0.5 M urea, 0.14 M 2-mercaptoethanol, 0.05 M sodium phosphate buffer pH 7.2	30 min, 37° then 1 min, 100°	0.1% SDS, 0.5 M urea, 0.014 M 2-mercaptoethanol	0.05 M iodoacetamide
4	As method 3, except that 0.1% dithiothreitol was present	10 min, 80°	As method 3	None

AA). Virus suspended in MEM-AA was adsorbed to the cells for 30 min on a roller drum at room temperature. The inoculum was replaced by prewarmed MEM-AA supplemented with 5% dialyzed fetal calf serum. The cultures were replaced at 37° and timing started from this point. Such cultures could then be labeled at any time by addition of the appropriate isotope diluted in MEM-AA. Labeling was terminated by flooding the cultures with ice-cold buffered saline.

Preparation of viral polypeptides for electrophoresis. Samples of pure virus or infected cells were incubated with SDS and urea in the presence of reagents that attack disulfide bonds as shown in Table 1. The samples were then dialyzed at room temperature prior to electrophoresis.

Electrophoresis of polypeptides. Polypeptide mixtures were analysed on 5% polyacrylamide gels as described by Haslam *et al.* (1969). Electrophoresis of radioactive samples was for 19.2 hours at 50 V; the gels were then fractionated into approximately 70 fractions. Electrophoresis of nonradioactive samples was for about 14 hours; the gels were then stained for protein using Coomassie brilliant blue. As a result of the briefer electrophoresis, the individual polypeptides were less well separated but the bands were less diffuse and consequently more suitable for detection by staining. Such gels were sometimes additonally stained for carbohydrate using the periodic acid–Schiff (PAS) reagent as described by Clarke (1964). After treatment with periodic acid it was necessary to leach out the oxidation products overnight before staining with basic fuchsin.

RESULTS

Purification of Influenza Virus

Influenza virus was extensively purified as described in the Materials and Methods. The final tartrate gradient was analysed to provide an indication of the degree of purity achieved (Fig. 1). Two peaks of coincident hemagglutinating activity and radioactivity were detected regardless of whether the initial step in the purification procedure had been ammonium sulfate precipitation or erythrocyte adsorption-elution. The major peak contained infectious virus with an average density of 1.19 g/ml, whereas the
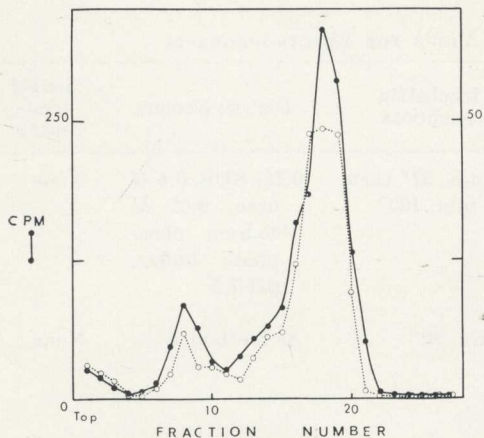
FIG. 1. Equilibrium gradient centrifugation of influenza A/Bel from calf kidney cells. Semipurified ^{14}C-amino acid-labeled virus from the penultimate sucrose gradient was centrifuged on a preformed gradient of 20–40% (w/v) potassium tartrate for 3 hours at 174,000 g in an SW 41 Spinco rotor. Fractions were analysed for radioactivity and hemagglutinating activity.

minor peak contained noninfectious hemagglutinating material of comparable sedimentation coefficient but lower density. Hence the equilibrium gradient was a vital step in removing contaminating membranous material that proved inseparable from the virus by rate zonal centrifugation. The complete purification procedure was applied to all preparations of the A/Bel strain and to nonradioactive samples of the B/Lee strain. However the final tartrate gradient was omitted from the purification of radioactive preparations of the B/Lee strain because of shortage of material; consequently polyacrylamide gels of dissociated Lee virus had a somewhat higher background of contaminating polypeptides.

The Polypeptides of Influenza A/Bel Virus from Calf Kidney Cells

Purified Bel virus which had been grown in calf kidney cells in the presence of ^{3}H-labeled valine and ^{14}C-labeled glucosamine was dissociated by a mild dissociation procedure (method 1 in Materials and Methods), and the polypeptides were analysed by polyacrylamide gel electrophoresis. The virus invariably yielded three major ^{3}H-

labeled electrophoretic peaks VP1, VP2, and VP3, and frequently gave two minor components, VP1a and VP1b, the latter being more prominent in stored preparations of virus (Fig. 2). Carbohydrate was present in VP1 and in both the minor components, VP1a and VP1b, as demonstrated by the incorporation of ^{14}C-labeled glucosamine. No trace of ^{14}C was evident in the other two major peaks, VP2 and VP3.

The Polypeptides of Influenza A/Bel Virus from Embryonated Hen's Eggs

To compare the effect of the host cell on the viral polypeptide pattern, Bel virus was also grown in hen's eggs. Coelectrophoresis of purified virus with Bel grown in calf kidney cells showed that VP1 and VP1b migrated faster when derived from egg-grown Bel, whereas the migration of VP2 and VP3 was unchanged (Fig. 3). The presence of carbohydrate in VP1a, VP1, and VP1b was detected by staining gels of egg-grown Bel with the periodic acid–Schiff (PAS) reagent as well as with Coomassie blue (Fig. 3). Incidentally, the fact that the five bands detected by staining with Coomassie blue correspond with the peaks obtained from radioactive virus preparations indicates that the purified virus was not contaminated with
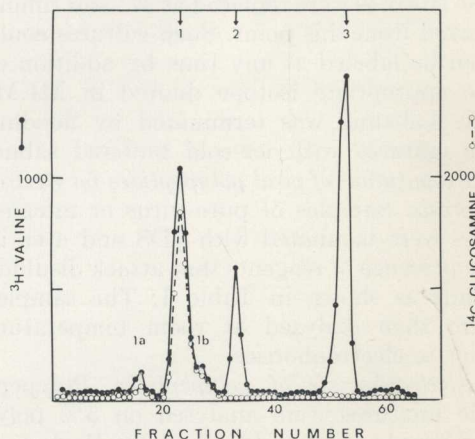

FIG. 2. The polypeptides of influenza A/Bel from calf kidney cells. Purified virus, prelabeled with valine-^{3}H and glucosamine-^{14}C was dissociated by method 1 and the resultant polypeptides were analyzed by polyacrylamide gel electrophoresis. Migration in this and all subsequent diagrams is towards the anode on the right.
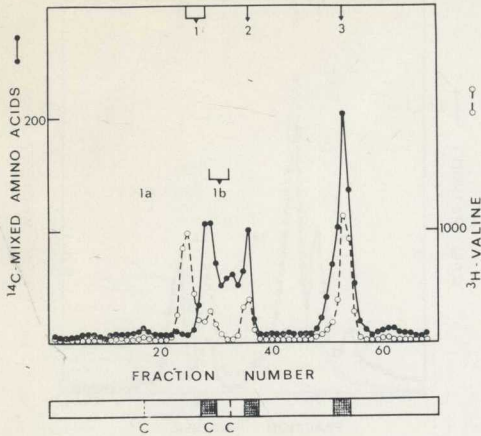
Fig. 3. The polypeptides of influenza A/Bel purified from hen's eggs. Bel virus grown in eggs (^{14}C-label) was coelectrophoresed with Bel virus from calf kidney cells (^{3}H-label) after dissociation by method 1. The lower panel represents the protein bands detected in Coomassie blue-stained gels of Bel from eggs. Bands that also stained for carbohydrate with the PAS reagent are labeled c.

detectable host proteins, and that it contains no significant quantity of preformed host protein. However, the possible inclusion in the virion of host cell proteins formed subsequent to virus infection cannot be unequivocally excluded.

Effect of Different Dissociation Conditions

The polypeptide patterns discussed so far were obtained by electrophoresis of virus which had been dissociated by method 1. When even more rigorous variations on this procedure (Table 1), involving alterations in pH, time or temperature of treatment, or in reducing conditions, were tested, it was found that the electrophoretic pattern of polypeptides from Bel grown in either eggs or calf kidney cells could be altered. Under certain conditions VP1 was labile, disaggregating or breaking down to yield material of lower molecular weight, hence greater electrophoretic mobility. Typical electrophoretic patterns obtained by the most rigorous dissociation procedure (method 2) are illustrated in Fig. 4.

VP1 of Bel grown in eggs was broken down by method 2 to give another glycoprotein migrating in the region of VP1b; an even smaller glycoprotein, which migrated adjacent to VP3, was resolved on stained gels. By contrast, the radioactivity from VP1 of calf kidney-grown Bel was distributed throughout the gel between VP1 and VP3. No significant overall loss of radioactivity occurred in either instance. Peaks VP2 and VP3 were completely unaffected by these changes in dissociation conditions. Repro-
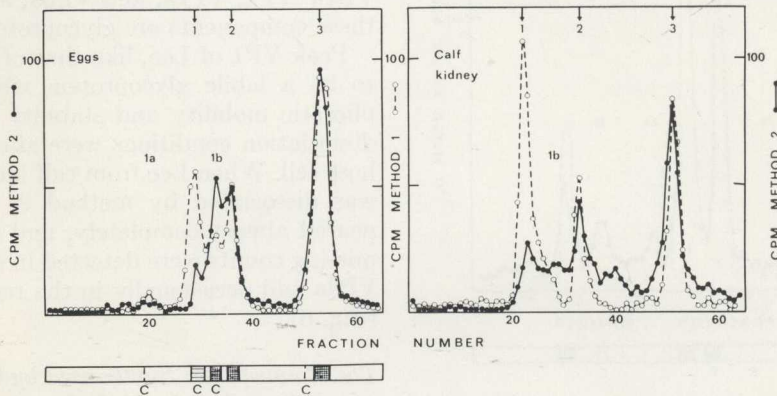

Fig. 4. The polypeptides of influenza A/Bel dissociated by method 2. Equal aliquots of ^{14}C-amino acid-labeled Bel virus, grown in eggs or calf kidney cells, were dissociated by either method 1 or method 2 and analysed by electrophoresis on parallel gels. The data from each pair of gels have been normalized to give equal total radioactive counts per gel. The actual cpm recovered from each gel were 860 and 847 for egg-grown Bel virus dissociated at pH 7.2 and 10.6, respectively, and 553 and 532 for calf kidney-grown Bel dissociated at pH 7.2 and 10.6, respectively. The lower panel represents the protein bands detected in Coomassie blue-stained gels of egg-grown Bel, dissociated by method 2. Bands that also stained with the PAS reagent are labeled c.

ducible results were obtained with three preparations of virus from each type of host cell. After storage for some months at $-70°$, Bel from eggs yielded an electrophoretic pattern typical of method 2 even when dissociated by method 1.

Dissociation procedures 3 and 4 (Table 1) broke down the VP1 of egg-grown Bel to give an electrophoretic pattern similar to that of method 2, but failed to affect the VP1 from calf kidney-grown Bel. The host cell therefore affects the stability of VP1 towards different dissociation conditions.

The Polypeptides of Influenza B/Lee Virus

Lee virus from eggs or calf kidney yielded at least six distinct electrophoretic peaks when dissociated by method 1. Coelectrophoresis of Lee and Bel grown in calf kidney cells indicated that two of the major components of Lee migrated with VP1 and VP3 of Bel, whereas another migrated slightly more slowly than VP2 (Fig. 5). For this reason the three major components of Lee were designated VP1, VP2, and VP3, while
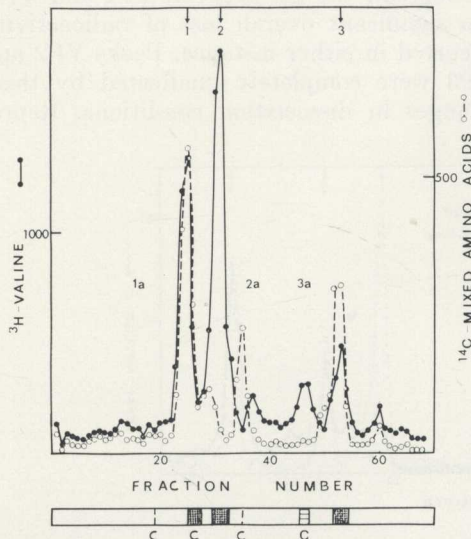
FIG. 6. The polypeptides of influenza B/Lee dissociated by method 2. Equal aliquots of ^{14}C-amino acid-labeled egg-grown Lee virus were dissociated by either method 1 or method 2, and analysed by electrophoresis on parallel gels. The data from the two gels have been normalized to give equal total radioactive counts per gel. The actual counts recovered were 1170 (method 1) and 1010 (method 2).

the minor components were labeled VP1a, VP2a, and VP3a. Peak VP2a was scarcely evident in radioactive samples but was resolved as a minor band close to VP2 in stained preparations of egg-grown virus. The PAS reagent regularly stained bands VP1a, VP1, VP2a, and VP3a, showing that these components are glycoproteins.

Peak VP1 of Lee, like that of Bel, proved to be a labile glycoprotein whose electrophoretic mobility and stability to different dissociation conditions were affected by the host cell. When Lee from calf kidney or eggs was dissociated by method 2, VP1 disappeared almost completely, and most of the missing counts were detected in glycoprotein VP3a and occasionally in the region of VP2 (Fig. 6).

The Polypeptides Synthesized by Calf Kidney Cells Infected with Influenza A/Bel

The recognition of VP1 as a glycoprotein raised the possibility that a carbohydrate-free polypeptide of lower molecular weight may first be synthesized in infected cells. Accordingly, the polypeptides synthesized in Bel-infected calf kidney cells were compared

FIG. 5. The polypeptides of influenza B/Lee. Valine-^{3}H labeled Lee virus from calf kidney cells was coelectrophoresed with ^{14}C-amino acid-labeled calf kidney-grown Bel after dissociation by method 1. The lower panel represents the protein bands detected in stained gels of Lee from eggs. Bands that also stained with the PAS reagent are labeled c.
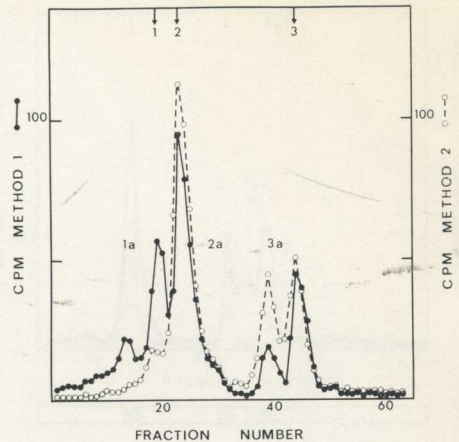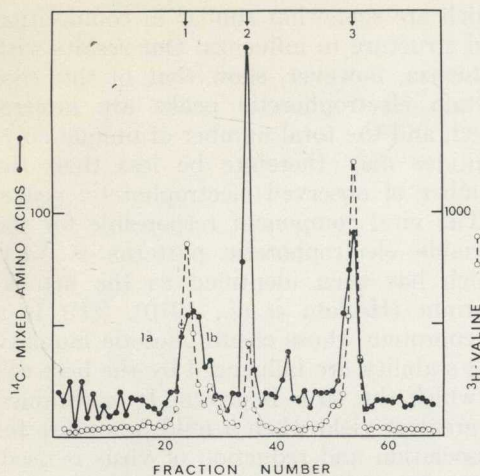
FIG. 7. The polypeptides synthesized by calf kidney cells infected with influenza A/Bel. The cells were incubated with ^{14}C-labeled mixed amino acids from 3.5 to 6.5 hours after infection with Bel virus. A cell sample was then added to valine-^{3}H-labeled Bel virus, which had been purified from calf kidney cells. The mixture was dissociated by method 1 and analysed by electrophoresis.

with those of the purified virus (Fig. 7). Method 1 was selected as the dissociation procedure in this experiment since it does not break down Bel VP1 from calf kidney cells into smaller glycoproteins which would complicate the search for a new low molecular weight polypeptide corresponding to the VP1 precursor (Fig. 7). Fully half of all the protein synthesized by calf kidney cells infected at high multiplicity coincided with the three major components of the virion, and no other prominent peaks were discernible. Even labeling periods as short as 3 min, followed by chases with nonradioactive amino acids for periods of 1–60 min in both calf kidney cells and HeLa cells provided no evidence for the existence of a precursor that chased into VP1 (Taylor et al., 1969).

In order to determine whether any of the electrophoretic peaks were themselves related as precursor and product, the amino acid composition of each was studied by the incorporation of different radioactive amino acids. The relative incorporation of selected amino acids into the three major viral peaks is shown in Table 2. Although, by this criterion, VP2 and VP3 were similar in com-

position, being relatively rich in arginine and methionine, VP1 was clearly different, being relatively enriched with cysteine. Therefore it appears to be most unlikely that VP2 or VP3 mature into VP1, or arise by posttranslational cleavage of VP1.

Viral Polypeptides Synthesized in Other Cell Systems

The viral polypeptides synthesized in a number of cell types other than calf kidney were also analysed. Additional systems investigated included primary chick embryo fibroblasts, a continuous line of African green monkey kidney cells (VERO), and the Wong-Kilbourne variant, clone 1-5C-4, of the continuous Chang human heteroploid conjunctival cell line (Wong and Kilbourne, 1961). Three major viral polypeptides were synthesized regardless of whether the cell system supported the production of infectious virions or only a nonproductive (abortive) cycle. The relative amounts of each peak were fairly constant from one cell line to another. In all cases VP2 was produced in excess compared with the proportion in purified virus. The electrophoretic mobility of VP2 and VP3 did not vary from one cell type to another, but coelectrophoresis of infected 1-5C-4 cells with infected calf kidney cells revealed minor differences in the mobility of VP1 (Fig. 8).

TABLE 2

RELATIVE INCORPORATION OF SELECTED AMINO ACIDS INTO BEL POLYPEPTIDES IN CALF KIDNEY CELLS[a]

Amino acid	VP1	VP2	VP3
Mixed	1.0	1.0	1.0
Arginine	1.0	2.2	1.8
Lysine	1.0	1.0	1.0
Cysteine	1.0	0.5	0.4
Methionine	1.0	3.5	3.3
Glutamic acid	1.0	1.1	1.1

[a] Cells infected 3.5 hours earlier were incubated with different ^{14}C-labeled amino acids for a further 3 hours. The amount of radioactivity incorporated into VP1, VP2, or VP3 was expressed relative to the incorporation of mixed ^{14}C-amino acids into the corresponding peak, then normalized so that this figure for VP1 = 1.0. Values given are the mean of at least two experiments.
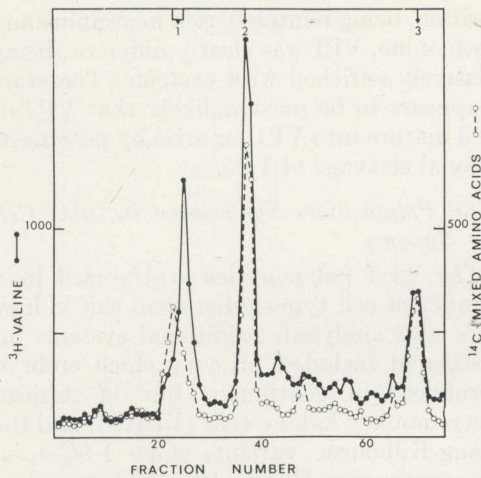
Fig. 8. The polypeptides synthesized by 1-5C-4 cells infected with influenza A/Bel. The cells were incubated with ^{3}H-labeled valine from 4–6 hours after infection with Bel virus. Infected calf kidney cells were similarly incubated with ^{14}C-labeled mixed amino acids. Samples of both were mixed, dissociated by method 1, and analysed by electrophoresis.

To test the possibility that the slightly greater electrophoretic mobility of VP1 in 1-5C-4 cells reflected a defect in glycoprotein biosynthesis in this cell line, the incorporation of glucosamine-^{14}C was studied. Since VP1 specifically incorporated this label, carbohydrate is present in the molecule, although not necessarily in the same amount as in calf kidney cells. Hence the dependence of the electrophoretic mobility of VP1 on the nature of the host cell is considered to reflect differences in the attached carbohydrate group.

DISCUSSION

This work began as an attempt to discover the total number of polypeptides in the influenza virion. The polyacrylamide gel electrophoresis system was chosen as a suitable analytical method because it gives reproducible results with other viruses. In particular, consistent results showing three major electrophoretic peaks have been obtained by different workers with Newcastle disease virus (Evans and Kingsbury, 1969; Haslam et al., 1969; Bikel and Duesberg, 1969) and with vesicular stomatitis virus (Wagner et al., 1969; Kang and Prevec, 1969)

which are somewhat similar in composition and structure to influenza. Our results with influenza, however, show that in this case certain electrophoretic peaks are interrelated, and the total number of unique polypeptides may therefore be less than the number of observed electrophoretic peaks.

The viral component responsible for the variable electrophoretic patterns is VP1, which has been identified as the hemagglutinin (Haslam et al., 1970). VP1 is a glycoprotein whose electrophoretic mobility and stability are influenced by the host cell in which the virus is grown. It is obtained in greatest yield when a mild procedure for dissociation and reduction of virus is used. After the application of more rigorous methods it diminishes or vanishes, while certain other electrophoretic peaks, namely VP1b and possibly a component of VP3 of Bel, and VP3a and possibly a component of VP2 of Lee, increase.

These observations explain the discrepancies in the literature with regard to the number and identification of influenza polypeptides. Two simple tests facilitate direct comparison of electropherograms of any given influenza strain obtained in different laboratories. Firstly, staining or labeling for carbohydrate provides an early indication of which major component is likely to be associated with the hemagglutinin. Secondly, comparison of different methods of dissociating the virion prior to electrophoresis will identify VP2 and VP3 as the stable polypeptides that are uninfluenced by these variations.

VP2 and VP3 have similar contents of the five amino acids tested, and in particular are enriched in methionine and arginine relative to VP1. However, the possibility that VP2 is a polymer of VP3 can probably be excluded on the grounds that both peaks are completely stable intracellularly following a short pulse-label and prolonged chase (Taylor et al., 1969), do not break down even under the most rigorous dissociation conditions (Table 1, methods 2, 3, 4), and appear to have different functions within the virion (Haslam et al., 1970).

The minor glycoprotein peak VP1a in both Bel and Lee is not related to VP1 by break-

down and probably represents a fourth unique viral component. Lee virus contains a further minor glycoprotein component VP2a which may be a fifth component, or may be a disaggregation product of VP1a (Haslam *et al.*, 1970).

The fact that, for a given strain of virus, the electrophoretic mobility of VP1 varies with the type of host cell in which the virus was grown suggests that the carbohydrate moiety of this molecule may be determined and assembled by the host cell's enzymes in the same way as for cellular glycoproteins. Hence pure virus lacking host protein could nevertheless carry a host cell antigen. A polysaccharide host cell antigen associated with the hemagglutinin has been demonstrated in influenza viruses grown in hen's eggs but was not demonstrable in viruses from calf kidney cells (Laver and Webster, 1966; Harboe, 1963; Haukenes *et al.*, 1966). However, as mentioned by Laver and Webster (1966), their results did not rule out the possibility that virus grown in calf kidney cells incorporates host carbohydrate which for some reason is not antigenic, or not directly concerned in the hemagglutinating site.

Detailed analysis of the isolated hemagglutinin is required to elucidate the relationship of VP1 to the glycoproteins of greater electrophoretic mobility derived from it. VP1 may be an aggregate of two or more, identical or different glycoprotein species which resists the milder methods of virus dissociation and reduction. This possibility is supported by the fact that VP1 is relatively rich in cysteine, suggesting that disulfide bonds may stabilize the structure; by the observations of Eckert (1966, 1967) that an influenza envelope protein may be disaggregated to 2 S subunits; and by genetic evidence that two cistrons may be involved in the hemagglutinin function (Mackenzie, 1968). An analogous situation occurs with polyoma virus capsid protein which contains resistant disulfide bonds (Fine *et al.*, 1968; Hare and Chan, 1968).

It is conceivable that the breakdown of VP1 is caused by β-elimination of certain carbohydrate groups attached to serine or threonine, resulting in peptide chain cleavage (Neuberger *et al.*, 1966; Adams, 1965; Witkop

1961). However, the fact that VP1 from virus grown in eggs is broken down by methods which do not include exposure to alkali provides a strong argument to the contrary.

The major electrophoretic peaks detected in Bel-infected calf kidney cells were identical with the components of pure virus, even when very short labeling times were employed. No polypeptide precursor (either larger or smaller) of the glycoprotein VP1 has yet been identified. The carbohydrate antigen from virus grown in eggs has a molecular weight of approximately 15,000 (Laver and Webster, 1966). If a comparable amount of carbohydrate is attached in other cell systems the difference in molecular weight between glycoprotein and polypeptide precursor should be sufficient to discriminate between them by gel electrophoresis. It is conceivable that the precursor comigrates with VP2 or VP3, or that assembly of the mature glycoprotein follows rapidly after synthesis of the polypeptide. Studies of nascent polypeptides on polyribosomes may clarify this point.

The biological role of carbohydrate in glycoproteins is not understood, although Eylar (1965) has suggested that the carbohydrate promotes the passage of the glycoprotein across cell membranes. In this respect it is significant that Bel VP1 is found in the plasma membrane of infected cells (White *et al.*, 1970). A surface protein of dengue virus (Stollar, 1969), the envelope proteins of Sindbis and vesicular stomatitis viruses (Burge and Strauss, 1970), and the hemagglutinin of Newcastle disease virus (Haslam, unpublished results) are all glycoproteins. The attachment of carbohydrate may be an important step in the maturation of these proteins at the plasma membrane of the cell.

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The Polypeptides of Influenza Virus

III. Identification of the Hemagglutinin, Neuraminidase and Nucleocapsid Proteins[1]

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The hemagglutinin and nucleocapsid protein of the A/Bel strain of influenza virus, and the neuraminidase of the B/Lee strain have been isolated and analysed by polyacrylamide gel electrophoresis in the presence of neutral sodium dodecyl sulfate. The hemagglutinin subunit is a glycoprotein with a molecular weight in the region of 77,000. The nucleocapsid protein has been identified as a polypeptide of molecular weight 50,000. The neuraminidase is composed of a polypeptide of molecular weight 56,000, and possibly a second polypeptide.

One of the major components of both viruses is a polypeptide of molecular weight 21,000, which corresponds to none of the known viral proteins.

INTRODUCTION

Influenza virus consists of a core of ribonucleoprotein surrounded by a complex lipoprotein envelope which is acquired during maturation at the cell surface (Compans and Dimmock, 1969; Bächi *et al.*, 1969). The external surface of the viral envelope is studded with at least two different types of protein subunit which are responsible for the hemagglutinating and neuraminidase activities of the virion, respectively (Laver and Valentine, 1969). Recent electron micrographs have suggested that the internal surface of the envelope is lined with an electron dense layer which may also be a protein (Apostolov and Flewett, 1969; Kendal *et al.*, 1969).

The hemagglutinin, nucleocapsid, and neuraminidase have each been isolated and purified by a variety of methods which include treatment of the virus with detergents to liberate one or more of the different components (Laver, 1963, 1964; Laver and Valentine, 1969; Webster and Darlington, 1969; Kingsbury and Webster, 1969; Duesberg, 1969; Pons *et al.*, 1969), or treatment with proteolytic enzymes to liberate the neuraminidase specifically (Noll *et al.*, 1962; Drzeniek *et al.*, 1966, 1968). It is not clear whether these three proteins constitute the entire protein complement of the virion. A number of enzymes such as an adenosine diphosphatase and triphosphatase, and a hemolysin have been reported in preparations of purified influenza, but these activities may be derived in trace amounts from the host cell (Neurath, 1964; Robinson and Duesberg, 1968).

Purified influenza virus can be dissociated by the sodium dodecyl sulfate (SDS) method of Maizel (1969) to yield a number of polypeptide species, clearly separable by acrylamide gel electrophoresis. In the accompanying report (Haslam *et al.*, 1970) it is shown that the precise number of these electrophoretic components depends upon the extent to which the dissociation conditions used break down the glycoprotein VP1 into

species of lower molecular weight. The simplest electrophorogram is produced following a dissociation procedure that minimizes the breakdown of VP1. This procedure was used throughout the present paper, in which the component polypeptides of the nucleocapsid, hemagglutinin, and neuraminidase are identified.

MATERIALS AND METHODS

Most of the materials and methods used in this work have been described in Taylor et al. (1969) and Haslam et al. (1969, 1970).

Virus and cells. Stocks of the A/Bel (Burnet and Bull, 1943) and B/Lee (Francis, 1947) strains of influenza were maintained by growth in fertile hen's eggs. Radioactively labeled virus was obtained by propagation of either strain for 3 days in primary embryonic calf kidney cell monolayer cultures (Taylor et al., 1969) in the presence of either ^{3}H-labeled valine or ^{14}C-labeled mixed amino acids (purified *Chlorella* protein hydrolyzate). The virus was purified from allantoic fluid or from the calf kidney culture medium as described by Haslam et al. (1970).

Dissociation of virus and electrophoresis of polypeptides. Samples were suspended in 2% SDS, 0.5 M urea, 0.04 M 2-mercaptoethanol, and 0.1 M 2-hydroxyethyldisulfide in 0.01 M sodium phosphate buffer pH 7.2, and incubated at 37° for 30 min, then at 100° for 1 min. This procedure is designated method 1 (Haslam et al., 1970). After dialysis against 0.1% SDS, 0.5 M urea at pH 7.2, the samples were subjected to electrophoresis for 19.2 hours at 50 V on 5% polyacrylamide gels containing 0.1% SDS and 0.5 M urea at pH 7.2.

Hemagglutination assays. Titrations were carried out in plastic trays using fowl erythrocytes (Haslam et al., 1969).

Neuraminidase assays. The enzyme was assayed at 37° in 0.1 M Tris-maleic acid buffer pH 6.0 in the presence of 1 mM CaCl$_2$, using fetuin as a substrate (Haslam et al., 1969). Liberated N-acetylneuraminic acid was titrated by the 2-thiobarbituric acid method of Warren (1959) except that color was extracted into n-butanol containing 5% 12 N HCl (Aminoff, 1961). Since sucrose interferes with this assay, fractions from sucrose gradients were dialysed, or diluted at least 1:100 before assay.

TABLE 1

DISRUPTION OF INFLUENZA VIRUS BY DIFFERENT TREATMENTS[a]

Virus	Method of disruption	Total residual activity after disruption in pellet plus supernatant fractions (expressed as % of untreated control)		Activity in supernatant fraction (expressed as % of total residual activity in both fractions)	
		Hemag-glutinin	Neura-minidase	Hemag-glutinin	Neura-minidase
Bel	Treated with 1% sodium deoxycholate in 0.1 M Tris buffer pH 8.0 for 1 hour at 0°	72[b]	74	60	80
		43[b]	43	47	65
Bel	Treated with trypsin at 4 mg/ml in 0.1 M Tris buffer pH 8.0 for 15 min at 37°	104	110	4	1
Lee	Treated with trypsin at 2.5 mg/ml in 0.1 M sodium phosphate buffer pH 7.2 for 15 min at 37°	—	137	—	92
Lee	Treated with 2% SDS for 10 min at 25°	—	118	—	100

[a] Virus concentrated from allantoic fluid into 0.5 ml was treated as described above, diluted to 2 ml with buffered saline (0.85% NaCl in 0.01 M sodium phosphate buffer pH 7.2), and centrifuged at 100,000g for 60 min. The supernatant was removed, and the pellet was resuspended in buffered saline by ultrasonic vibration for 1 min. Both fractions were assayed for hemagglutinating and neuraminidase activities.

[b] Somewhat different results were obtained in the two experiments given.
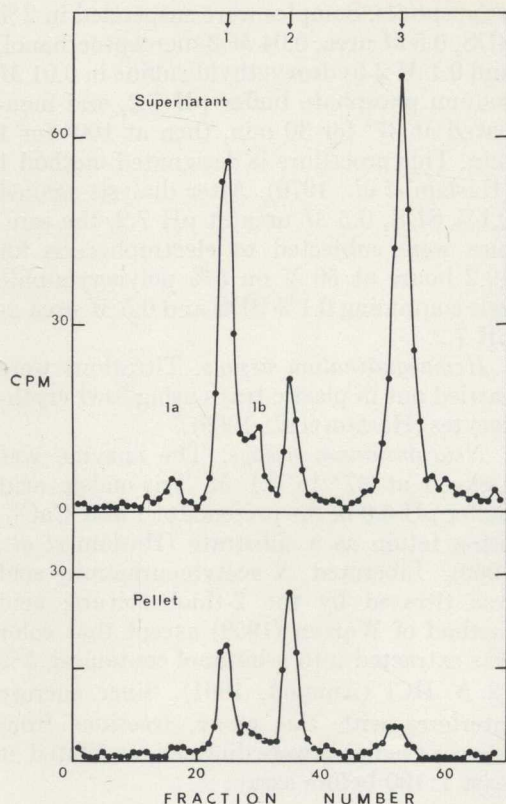
FIG. 1. Polypeptide composition of fractions from influenza A/Bel obtained by dissociation with deoxycholate. Bel virus was disrupted with sodium deoxycholate and centrifuged at 100,000 *g* for 60 min. The pellet and supernatant were analysed for hemagglutinin and neuraminidase (Table 1), then completely dissociated to their component polypeptides using method 1 (Haslam *et al.*, 1970). The polypeptides were separated by electrophoresis at pH 7.2 in 5% polyacrylamide gels containing 0.1% SDS and 0.5 *M* urea. A sample of purified Bel virus was similarly dissociated by method 1 and analysed by electrophoresis. Migration in this and all subsequent diagrams is towards the anode on the right. The positions of VP1, 2 and 3 from the marker virus are indicated by arrows.

RESULTS

Disruption of Influenza A/Bel Virus into Biologically Active Subunits

Several different methods of disrupting the Bel strain of influenza were tested for their ability to yield small subunits that still retained biological activity. Sodium deoxycholate liberated active hemagglutinin and

neuraminidase from a viral "core" which could be pelleted by centrifugation at 100,000 *g* for 60 min (Table 1). The pellet and supernatant were then dissociated to their component polypeptides (method 1, Haslam *et al.*, 1970) and analysed by acrylamide gel electrophoresis (Fig. 1).

Much of the VP1 and nearly all the VP3 of the virion was found in the material liberated into the supernatant by deoxycholate, indicating that these are relatively superficial proteins easily detached from the virion. By contrast, more than half of the VP2 was
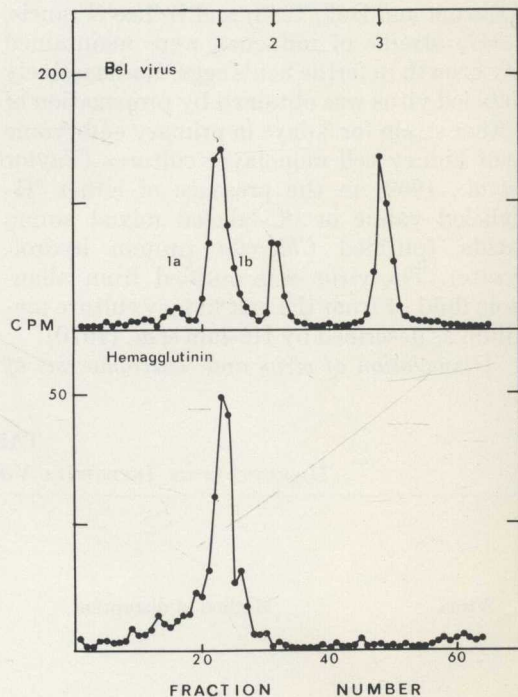

FIG. 2. Electrophoresis of Bel hemagglutinin, isolated from purified virus. The 100,000 *g* supernatant fraction from deoxycholate-disrupted Bel virus was dialysed at 4° for 3 days against several changes of buffered saline to remove the deoxycholate. Fowl erythrocytes were added to a final concentration of 2%, and the mixture was left at 4° for 5 min. The erythrocytes were collected by centrifugation and the adsorbed hemagglutinin was eluted by incubation with neuraminidase from *Vibrio cholerae* at 37° for 1 hour at pH 6.2 in the presence of 5 m*M* calcium chloride. The hemagglutinin was then dissociated using method 1 and analysed by electrophoresis in parallel with a sample of purified Bel virus.
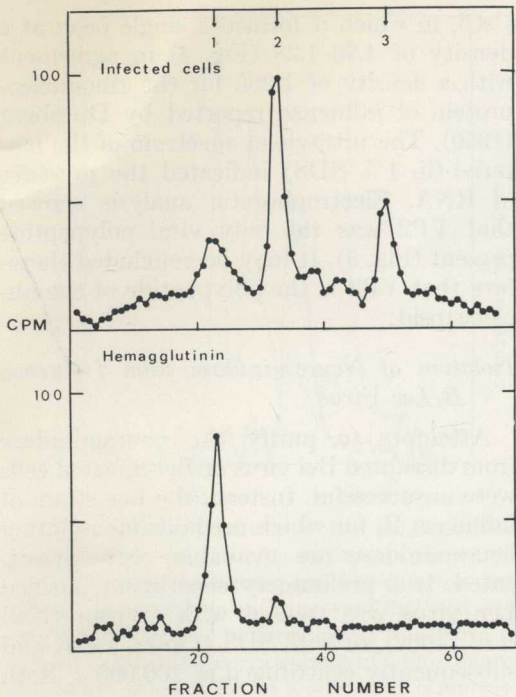
FIG. 3. Electrophoresis of Bel hemagglutinin, isolated from infected cells. Infected calf kidney cells were disrupted by ultrasonic vibration then centrifuged at 80,000 g for 40 min. Small hemagglutinating subunits which remained in the supernatant fluid were isolated by adsorption to fowl erythrocytes as described in the legend of Fig. 2. The isolated hemagglutinin was dissociated by method 1 and analysed by electrophoresis in parallel with a sample of the original infected cells.

found in the pellet, suggesting that VP2 may be the internal nucleocapsid protein.

Attempts to separate the hemagglutinating and neuraminidase activities in the disrupted preparation by gradient centrifugation proved unsuccessful because the material was very heterogeneous in size and density. However the hemagglutinin was successfully isolated from the supernatant fraction by adsorption to fowl erythrocytes, followed by elution with neuraminidase purified from *Vibrio cholerae*. Electrophoretic analysis showed that this material contained VP1, but no VP2 or VP3 (Fig. 2). VP1 was therefore strongly implicated as a component of the hemagglutinin.

Isolation of Hemagglutinin and Soluble Antigen from Bel-Infected Cells

Calf kidney cells in which Bel had been growing for 3 days in the presence of radioactive amino acids were also used as a source of viral components. The cells were disrupted by ultrasonic vibration, then virions, cell debris, and other large particulate matter were removed by centrifugation (80,000 g, 40 min). The supernatant retained only 2–5% of the original hemagglutinating activity, but this residual activity could be recovered in 80–100% yield by adsorption to fowl erythrocytes and subsequent elution with *Vibrio cholerae* neuraminidase. (Trace amounts of viral neuraminidase were also present in the supernatant fraction, but did not adsorb to the fowl erythrocytes.) The eluted hemagglutinin was analysed by electrophoresis (Fig. 3) and found to consist of
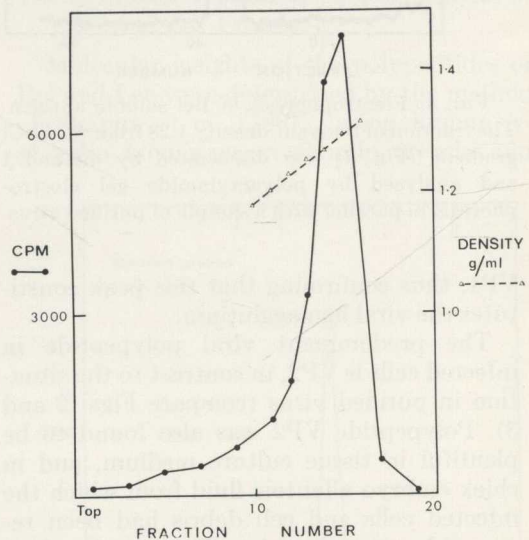

FIG. 4. Density gradient centrifugation of Bel soluble antigen. Culture medium from Bel-infected calf kidney cells was centrifuged at 64,000 g for 30 min to deposit virus particles. The supernatant fluid was then adjusted to pH 4.5 with 0.05 M acetate buffer, and the precipitate which formed overnight was isolated and resuspended at pH 7.2. The material was then dialysed overnight at 4° against buffered saline, clarified by a low speed centrifugation and layered onto a gradient of 11–38% (w/w) CsCl. After centrifugation at 180,000 g for 15 hours, the gradient was analysed for radioactivity and density.
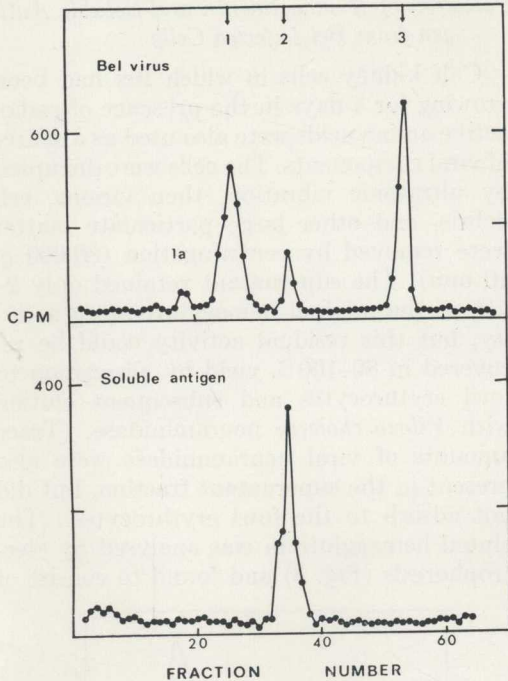
FIG. 5. Electrophoresis of Bel soluble antigen. The fraction of buoyant density 1.28 from the CsCl gradient (Fig. 4) was dissociated by method 1 and analysed by polyacrylamide gel electrophoresis in parallel with a sample of purified virus.

VP1, thus confirming that this peak constitutes the viral hemagglutinin.

The predominant viral polypeptide in infected cells is VP2, in contrast to the situation in purified virus (compare Figs. 2 and 3). Polypeptide VP2 was also found to be plentiful in tissue culture medium, and in chick embryo allantoic fluid from which the infected cells and cell debris had been removed by centrifugation. There is abundant evidence that the "soluble antigen" (S antigen) of influenza, which is antigenically identical with the nucleocapsid protein, is produced in excess during infection and accumulates in the culture medium (Frisch-Niggemeyer and Hoyle, 1956; Hoyle, 1952). Since the soluble antigen can be readily precipitated at pH 4.5 (Schäfer and Munk, 1952), this procedure was used to isolate the material both from the medium of infected cell cultures and from allantoic fluid of infected chick embryos. The precipitate was resuspended and purified on a gradient of

CsCl, in which it formed a single peak at a density of 1.26–1.28 (Fig. 4) in agreement with a density of 1.265 for the ribonucleoprotein of influenza reported by Duesberg (1969). The ultraviolet spectrum of the material (in 1% SDS) indicated the presence of RNA. Electrophoretic analysis showed that VP2 was the only viral polypeptide present (Fig. 5). It may be concluded therefore that VP2 is the polypeptide of the nucleocapsid.

Isolation of Neuraminidase from Influenza B/Lee Virus

Attempts to purify the neuraminidase from disrupted Bel virus or Bel-infected cells were unsuccessful. Instead, the Lee strain of influenza B, for which methods for isolating neuraminidase are available, was investigated. In a preliminary experiment purified Lee virus was treated with trypsin (Noll *et al.*, 1962), or with SDS (Laver, 1963) and subsequently centrifuged at 100,000 *g*. Both
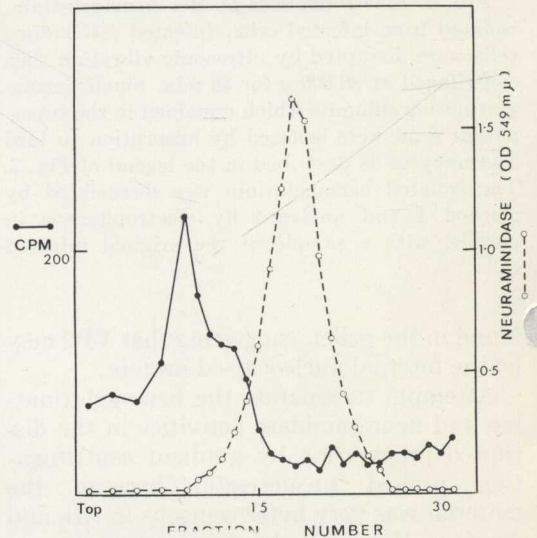

FIG. 6. Sucrose gradient centrifugation of Lee neuraminidase, isolated from the virion by SDS treatment. Radioactive Lee virus purified from calf kidney cells was supplemented with nonradioactive Lee purified from egg allantoic fluid and treated with SDS (2%) for 10 min at 25°. The mixture was layered onto a gradient of 5–20% (w/v) sucrose, centrifuged at 20° for 15 hours at 81,000 *g*, and then analysed for neuraminidase activity and radioactivity. Fractions 15–23 were then pooled.
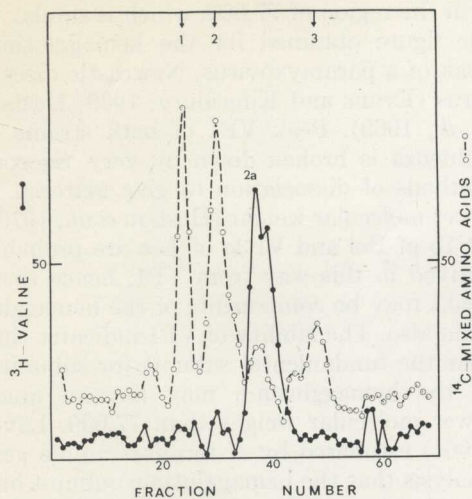
FIG. 7. Electrophoresis of Lee neuraminidase, isolated from the virion by trypsin treatment. The fractions from the sucrose gradient showing maximum neuraminidase activity were dialysed overnight at 4° against buffered saline to remove the sucrose, and reduced to a volume of approximately 0.2 ml by evaporation in a stream of compressed air. The neuraminidase (valine-^{3}H) was then dissociated by method 1 and analysed by coelectrophoresis with a sample of purified Lee virus (^{14}C-mixed amino acids).

treatments caused a small increase in total neuraminidase activity (assayed using fetuin as substrate), 90–100% of which was found in the supernatant fraction (Table 1). Enzyme liberated from the virus by either method was then purified by sucrose gradient centrifugation and analysed by polyacrylamide gel electrophoresis.

The purification of the neuraminidase from SDS-treated Lee is shown in Fig. 6. The enzyme activity sedimented ahead of most of the radioactivity, which remained at the top of the gradient. Neuraminidase liberated from Lee by trypsin sedimented at a comparable rate, but was more clearly separated from the remainder of the radioactivity, most of which, in this case, remained associated with a rapidly sedimenting viral "core."

Those fractions from the gradient which contained neuraminidase were pooled, dissociated by method 1, and analysed by electrophoresis together with purified Lee virus as a marker (Figs. 7–9). As is usual for Lee

(Haslam et al., 1970) the marker virus gave three major electrophoretic components, VP1, VP2, and VP3, and three minor components, VP1a, VP2a, and VP3a. Neuraminidase isolated from calf-kidney-grown Lee by trypsin treatment migrated at the same rate as VP2a (Fig. 7). As this was the major electrophoretic component detected in several such experiments, polypeptide VP2a must contain the active site of the enzyme. By contrast, the neuraminidase isolated from SDS-treated Lee contained variable amounts of VP1a and VP2, in addition to VP2a. Two such preparations are shown in Figs. 8 and 9. If the enzyme preparation was not dissociated by method 1, but subjected directly to electrophoresis, VP1a was found to be the predominant component, suggesting that VP1a is a polymer.

The Molecular Weights of the Polypeptides of Bel and Lee Viruses

Molecular weights of the polypeptides of Bel and Lee were determined by the method of Shapiro et al. (1967), using human γ-globulin, bovine serum albumin, trypsin, and
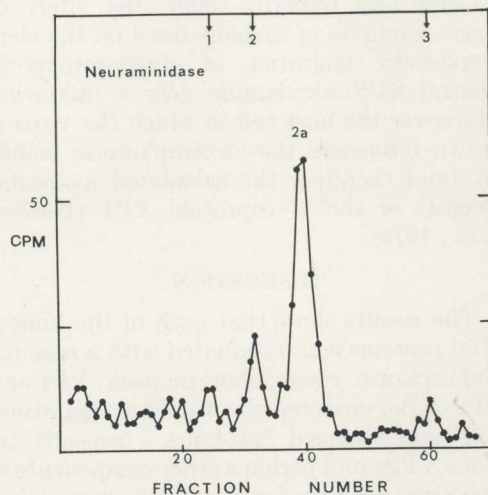

FIG. 8. Electrophoresis of Lee neuraminidase, isolated from the virion by SDS treatment. The fractions from the sucrose gradient (Fig. 6) showing maximum neuraminidase activity were dialysed at 4° against buffered saline to remove sucrose and SDS, and concentrated by evaporation. The material was then dissociated by method 1 and analysed by polyacrylamide gel electrophoresis.
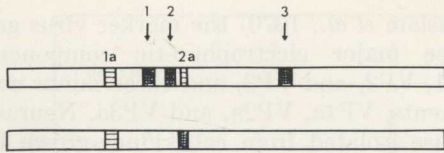
FIG. 9. Electrophoresis of Lee neuraminidase, isolated from the virion by SDS treatment. Purified Lee, which had been grown in eggs, was treated with 2% SDS. The liberated neuraminidase was isolated as shown in Fig. 8 and analysed by polyacrylamide gel electrophoresis in parallel with a sample of virus (top gel).

cytochrome c as marker proteins. The figures in Table 2 refer to the polypeptide bands visible in stained gels of samples of both viruses purified from allantoic fluid. All the molecular weights are within the range 20,000–135,000. There are some differences between the two strains of virus, particularly in the figures for VP2. The values estimated for those peaks which contain carbohydrate must be viewed with caution, for although this method has been proved accurate in determining the molecular weights of a few glycoproteins, such as ovalbumin, which contain no more than 5% of carbohydrate (Weber and Osborn, 1969), the effect of larger amounts of carbohydrate on the electrophoretic migration of glycoproteins in neutral SDS acrylamide gels is unknown. Moreover the host cell in which the virus is grown influences the electrophoretic mobility (and therefore the calculated molecular weight) of the glycoprotein VP1 (Haslam et al., 1970).

DISCUSSION

The results show that each of the known viral proteins can be equated with a specific, and separate, electrophoretic peak. VP1 and VP2 of Bel virus represent the hemagglutinin and nucleocapsid proteins, respectively, while VP2a and perhaps other components of Lee virus are associated with the neuraminidase.

The hemagglutinin peak, VP1, of Bel is a glycoprotein that contains approximately 40% of the total amino acid label in the virion. VP1 of Lee virus is also a glycoprotein and presumably corresponds to the hemagglutinin of this influenza B strain. The molecular weight of VP1 of both virus strains

is in the region of 77,000, which is similar to the figure obtained for the hemagglutinin peak of a paramyxovirus, Newcastle disease virus (Evans and Kingsbury, 1969; Haslam et al., 1969). Peak VP1 of both strains of influenza is broken down by very rigorous methods of dissociation to give material of lower molecular weight (Haslam et al., 1970). VP1b of Bel and VP3a of Lee are probably derived in this way from VP1, hence these peaks may be components of the hemagglutinin also. The lability of VP1 indicates that that the fundamental subunit (or subunits) of the hemagglutinin may have a much lower molecular weight than 77,000. Laver (1964) estimated by N-terminal amino acid analysis that the hemagglutinin subunit had a molecular weight of the order of 60,000. However, exclusion chromatography of rigorously dissociated hemagglutinin suggested a figure of 47,000 (Webster, 1970). These discrepancies probably arise from the complex structure of the hemagglutinin and will be resolved by further work.

The intact, undissociated form of the hemagglutinin has been isolated from in-

TABLE 2

MOLECULAR WEIGHTS OF THE POLYPEPTIDES OF BEL AND LEE GROWN IN EGGS[a]

Electrophoretic peak	Molecular weight	
	Bel	Lee
VP1a	133,000	106,000
VP1	76,500	78,000
VP2	50,000	63,000
VP2a	—	56,000
VP3a[b]	—	27,000
VP3	21,000	21,000

[a] Purified Bel and Lee were dissociated by method 1. The polypeptides were separated by electrophoresis for 14 hours at 6 mA per gel, in parallel with the following marker proteins, human γ-globulin, bovine serum albumin, trypsin, and cytochrome c. The gels were stained with Coomassie brilliant blue, and the molecular weights of the viral bands were estimated by comparison with the marker proteins (Shapiro et al., 1967; Weber and Osborne, 1969).

[b] VP3a was not always evident on the stained gels; its molecular weight was extrapolated from its migration relative to the other viral peaks in radioactive samples.

fluenza virus as rodlike structures with a sedimentation coefficient of 7.8–8.1 S and dimensions of 14 x 4 nm (Laver and Valentine, 1969; Webster and Darlington, 1969). The molecular weight of these rods, which correspond to the surface spikes of the virion, was estimated at 150,000; each spike may therefore be a dimer of the glycoprotein represented by VP1, or a polymer of a smaller molecule yet to be isolated.

The nucleocapsid protein, VP2, of the A/Bel strain of influenza has a molecular weight of 50,000 and constitutes approximately 15% of the total viral protein. Identical results in both respects were recently reported for the nucleocapsid protein of the A/WSN strain (Pons et al., 1969). In Bel-infected cells VP2 incorporates arginine with high efficiency (Haslam et al., 1970). This is consistent with a report that the soluble antigen contains 9.8% by weight of arginine (Hoyle and Davies, 1961). The core proteins of the adenovirion are also rich in arginine, which may be required to neutralize charges on the viral nucleic acid (Maizel et al., 1968). VP2 of the B/Lee strain has a somewhat higher molecular weight and constitutes approximately 30% of the viral protein. There is no evidence whether this peak includes the nucleocapsid protein of Lee virus, although it seems probable. In addition, it appears that a breakdown product of VP1 (Haslam et al., 1970) and perhaps a component of the neuraminidase (see below) may migrate with VP2 of Lee.

The polypeptide VP2a was found in preparations of neuraminidase isolated by treatment of purified virus with SDS or trypsin; it probably contains the active site of the enzyme. The additional peaks, VP1a and VP2, which were observed in neuraminidase obtained by SDS treatment, could conceivably represent contaminants, in view of the fact that these preparations were less well separated from most of the radioactivity on the sucrose gradient than was the trypsin-derived enzyme. Alternatively, it is possible that trypsin destroys part of the enzyme, leaving VP2a. Webster (1970) recently reported two electrophoretic components of similar molecular weight (about 58,000) in purified neuraminidase from influenza strain $A_2/RI/5^+$ together with some material of higher molecular weight, presumably representing undissociated polymer. Therefore VP2 and VP2a may correspond to Webster's adjacent neuraminidase bands, while VP1a corresponds to a higher molecular weight polymer.

When the neuraminidase is liberated from influenza virus by proteolytic enzymes or detergents, it has a sedimentation coefficient in the region of 8.5–10.8 S (Noll et al., 1962; Drzeniek et al., 1966; Laver, 1963; Laver and Valentine, 1969; Webster and Darlington, 1969). Because of its size the intact neuraminidase is clearly an aggregate of several polypeptides. The 8.5 S species isolated by Laver and Valentine (1969) has the appearance of a "mushroom," the "stalk" of which is believed to be attached to the viral membrane. Conceivably, trypsin treatment of Lee yields only the "head" of the mushroom, whereas SDS treatment yields the complete structure. Further work is required to investigate this possibility.

The remaining viral polypeptide, VP3, does not correspond to any known viral constituent, nor does it result from the breakdown of any other electrophoretic peak. VP3 has a very similar composition to VP2 as judged by the uptake of certain amino acids in Bel-infected cells (Haslam et al., 1970), which might point to it being a core protein. Moreover Joss et al. (1969) have shown that the equivalent peak in fowl plague-infected cells associates with the nucleocapsid protein in a cytoplasmic fraction. Yet the ease with which VP3 is separated from the nucleocapsid core by deoxycholate suggests that it is a fairly superficial protein. Recently the inner layer of the influenza envelope has been shown to have a characteristic subunit structure (Apostolov and Flewett, 1969; Kendal et al., 1969). VP3 may provide this link between the nucleocapsid core and the surface glycoproteins.

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The Polypeptides of Influenza Virus

IV. An Analysis of Nuclear Accumulation

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Two of the three major structural polypeptides of influenza virus A/Bel accumulate in the nucleus of cells that support the production of infectious virus as well as those in which the cycle is abortive. The migration is uninfluenced by multiplicity of infection, or by the addition of cycloheximide or actinomycin D.

The polypeptide of the nucleocapsid migrates relatively slowly, and is found mainly in the nucleoplasm, whereas the other arginine-rich polypeptide, which is of lower molecular weight, migrates very rapidly and is found mainly in the nucleolus.

Two of the three major polypeptides of the paramyxovirus, Newcastle disease virus, also accumulate in the nucleus.

INTRODUCTION

By means of polyacrylamide gel electrophoresis, it has been shown that purified influenza virus (strain A/Bel) contains three major constituent proteins (Taylor *et al.*, 1969). The first, VP1, is a glycoprotein identified as the viral hemagglutinin; the second, VP2, is the polypeptide of the nucleocapsid; the third, VP3, is a polypeptide of unknown function; the neuraminidase is a more minor constituent, separable from all three (Haslam *et al.*, 1970a,b). In HeLa cells infected at high multiplicity by influenza virus the three main viral proteins are synthesized in the cytoplasm. Subsequently VP2, and especially VP3, rapidly accumulate in the nucleus (Taylor *et al.*, 1969). The present communication reports an investigation into the basis of this phenomenon of nuclear accumulation.

MATERIALS AND METHODS

Most of the methods used in this work have previously been described (Taylor *et al.*, 1969); minor variations are noted in the text.

Fractionation of isolated nuclei. The method used is basically that of Penman *et al.* (1967). Purified nuclei from 2×10^7 cells are resuspended in 0.5 ml of "high salt" buffer (0.5 M NaCl, 0.05 M MgCl$_2$, 0.01 M Tris pH 7.4). The suspension is warmed to 37°, deoxyribonuclease is added to 50 μg/ml and the suspension is gently agitated for about 2 min, by which time the viscosity of the suspension is considerably reduced. The preparation is then chilled and centrifuged at 10,000 g for 5 min. The resulting supernatant will be referred to as "nucleoplasm," the pellet as the "nucleolar fraction."

Materials. Actinomycin D was a gift from Merck, Sharp and Dohme (West Point, Pennsylvania). Cycloheximide was obtained from Upjohn (Kalamazoo, Michigan).

RESULTS

Effect of Multiplicity on Nuclear Accumulation of Viral Polypeptides

The initial report of the nuclear accumulation of influenza viral polypeptides after their synthesis in the cytoplasm (Taylor *et al.*, 1969) involved the use of a very high multiplicity of infection (m.o.i.). The following experiment was designed to test whether the phenomenon occurs at lower m.o.i.
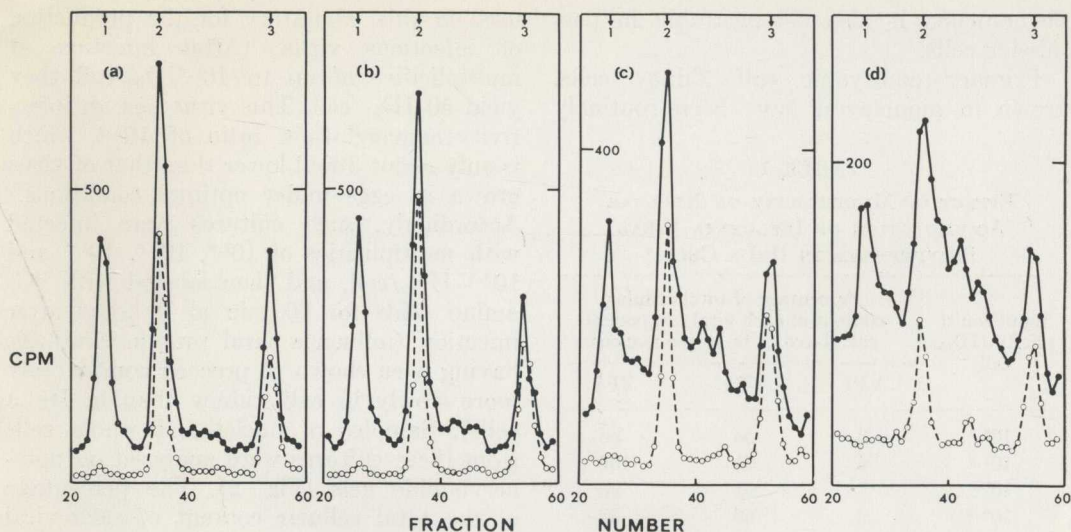
FIG. 1. Nuclear accumulation of viral polypeptides in HeLa cells infected with influenza virus at various multiplicities. Suspension cultures of HeLa cells were washed once, and resuspended at a concentration of 10^7 cells/ml in serum-free Minimal Essential Medium (MEM) (Eagle, 1959) that had been modified to contain one-tenth the usual concentration of amino acids. Aliquots of 2×10^7 cells were then incubated for 30 min at room temperature with influenza virus at multiplicities of (a) $10^{2.0}$, (b) $10^{1.5}$, (c) $10^{1.0}$, and (d) $10^{0.5}$ ID_{50}/cell. The suspensions were then diluted 10-fold with prewarmed MEM, lacking amino acids and supplemented with 5% dialysed fetal calf serum. After incubation at 37° with stirring for 3.5 hours, the cells were deposited by gentle centrifugation (250 g, 5 min) and resuspended in amino acid-free medium at a concentration of 4×10^7 cells/ml. After a 10 min recovery period, ^{14}C-labeled amino acids were added for 10 min. A chase of 50 min in the presence of unlabeled amino acids was achieved by diluting the cells 40-fold into complete MEM.

Cells were fractionated into nucleus and cytoplasm, and analyzed by electrophoresis in 5% polyacrylamide gels. Migration in this and all subsequent figures is toward the anode, on the right. Solid lines = whole cells; broken lines = nuclei. In each of the four panels, the electrophorogram of nuclei has been normalized so that the number of nuclei equals the number of whole cells analyzed on the accompanying gel (Taylor et al., 1969).

Suspension cultures of HeLa cells were inoculated with influenza A/Bel virus at input multiplicities of $10^{2.0}$, $10^{1.5}$, $10^{1.0}$, and $10^{0.5}$ ID_{50}/cell. At 3.8 hours after infection the cultures were labeled with ^{14}C-mixed amino acids for 10 min, then chased with excess unlabeled amino acids for 50 min. The cells were disrupted, separated into nuclear and cytoplasmic fractions, and analyzed by polyacrylamide gel electrophoresis (Fig. 1).

The electropherograms of whole cells demonstrate that the three major viral polypeptides, VP1, VP2, and VP3, are the dominant species synthesized at all m.o.i. except the lowest, where most of the cells are not infected. The purified nuclei, on the other hand, contain only VP2 and VP3 over the whole range of m.o.i. The percentage of

the total cellular content of each labeled viral polypeptide that is present in the nucleus at the end of the chase period is shown in Table 1.

At all m.o.i. most of the VP2 and VP3 is present in the nucleus at the end of the chase period.

Nuclear Accumulation in Permissive Cells

Because of the advantages of using continuous cell lines adapted to growth in suspension culture the initial investigations into nuclear accumulation of influenza polypeptides were conducted in suspended HeLa cells. However, such cells are "nonpermissive", in that infectious virus is not released (Henle et al., 1955). Consequently it was of interest to determine whether the

phenomenon is also demonstrable in permissive cells.

Primary embryonic calf kidney cells, grown in monolayer, have been routinely

TABLE 1

Effect of Multiplicity on Nuclear Accumulation of Influenza Viral Polypeptides in HeLa Cells[a]

Input multiplicity (ID$_{50}$/ cell)	Percentage of total cellular content of each viral component calculated to be in the nucleus		
	VP1	VP2	VP3
$10^{2.0}$	3	56	68
$10^{1.5}$	5	51	107
$10^{1.0}$	7	61	93
$10^{0.5}$	6	60	58

[a] The amount of each viral protein in the nucleus is expressed as a percentage of that in the whole cell. These figures were calculated from the data of Fig. 1. To estimate the amount of radioactivity in a peak of viral protein, it was assumed that all radioactivity falling below a line connecting the troughs on either side of that peak was due to cellular protein. This assumption involves only minor errors, as electrophorograms of uninfected cells are relatively smooth (Taylor *et al.*, 1969, Fig. 1c).

used in this laboratory for the production of infectious virus. (After infection at multiplicities of up to $10^{1.0}$ID$_{50}$/cell they yield 50 ID$_{50}$/cell. This virus has an infectivity:hemagglutinin ratio of $10^{5.0}$, which is only about 3-fold lower than that of virus grown in eggs under optimal conditions.) Accordingly, such cultures were infected with multiplicities of $10^{2.0}$, $10^{1.5}$, $10^{1.0}$ and $10^{0.5}$ ID$_{50}$/cell, and then labeled with ^{14}C-amino acids for 60 min at 9 hours after infection (influenza viral protein synthesis having been shown to proceed considerably more slowly in calf kidney than in HeLa cells). Samples of nuclei and whole cells from these cultures were analyzed on polyacrylamide gels (Fig. 2). The percentage of the total cellular content of each viral polypeptide present in the nucleus at the end of the labeling period is given in Table 2.

It can be seen that VP2 and VP3 are present in the nucleus of these permissive cells to an extent that does not vary significantly over the range of m.o.i. from $10^{2.0}$ to $10^{1.0}$ ID$_{50}$/cell. (At the lowest m.o.i., $10^{0.5}$, the precise amounts of these viral polypeptides present in the unfractionated cells are difficult to discern in the electro-
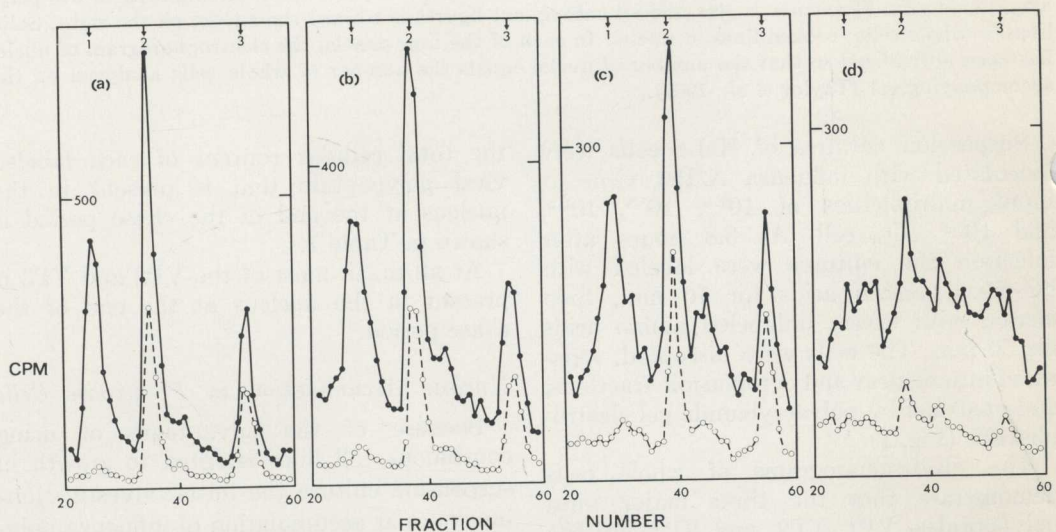

Fig. 2. Nuclear accumulation of viral polypeptides during productive infection of calf kidney cells by influenza virus. Monolayer cultures of primary embryonic calf kidney cells were exposed for 30 min at room temperature to influenza virus at multiplicities of (a) $10^{2.0}$, (b) $10^{1.5}$, (c) $10^{1.0}$, and (d) $10^{0.5}$ ID$_{50}$/ cell. At 9 hours after infection the cultures were labeled for 1 hour by the addition of ^{14}C-amino acids to the medium. The cells were fractionated and analyzed as described in the legend of Fig. 1. Solid lines = whole cells; broken lines = nuclei.

TABLE 2

Effect of Multiplicity on Nuclear Accumulation of Influenza Viral Polypeptides in Calf Kidney Cells[a]

Input multiplicity (ID_{50}/cell)	Percentage of total cellular content of each viral component calculated to be in the nucleus		
	VP1	VP2	VP3
$10^{2.0}$	5	50	70
$10^{1.5}$	7	47	64
$10^{1.0}$	7	42	69

[a] Data are derived from Fig. 2 by the method described in Taylor et al. (1969).

pherogram against the background of cellular polypeptides.)

Inhibition of Nuclear Accumulation

The following experiments were designed to test whether the nuclear accumulation of VP2 and VP3 is dependent upon continuing protein or RNA synthesis. In all experiments HeLa cell cultures, at 3.8 hours after infection with $10^{2.0}$ ID_{50}/cell, were labeled for 3 min with ^{14}C-amino acids and chased in the presence of an excess of unlabeled amino acids for either 5 or 60 min. In order to investigate any requirement for protein synthesis, cycloheximide (100 μg/ml) was added at the beginning of the chase period. Assays of trichloroacetic acid-precipitable radioactivity showed that under these conditions incorporation of radioactive amino acids was reduced to 11% of control values within 5 min. To investigate any requirement for DNA-dependent RNA synthesis, actinomycin was maintained at 10 μg/ml from 3.3 hours after infection. In this case assays showed that incorporation of radioactive uridine was reduced to 7% of control values by the time the chase commenced half an hour later. Samples of nuclei and whole cells were obtained and analyzed on polyacrylamide gels. The results are summarized in Table 3.

In the control cultures, nuclear accumulation of VP3 is seen to be very rapid, being almost complete within 5 minutes. VP2 accumulates more slowly. Neither actinomycin nor cycloheximide had any marked effect on the percentage of VP2 or VP3 present in the nucleus by 60 min.

TABLE 3

Effect of Inhibitors on Nuclear Accumulation of Influenza Viral Polypeptides[a]

Inhibitor	Duration of chase (min)	Percentage of total cellular content of each viral component calculated to be in the nucleus		
		VP1	VP2	VP3
Control	5	9	26	82
	60	4	49	81
Cycloheximide	5	6	12	46
	60	4	32	73
Actinomycin	5	7	9	60
	60	4	35	64

[a] Suspension cultures of HeLa cells at 3.8 hours after infection with 10^2 ID_{50}/cell were incubated for 3 min with ^{14}C-amino acids and then chased for either 5 or 60 min in the presence of an excess of unlabeled amino acids. The inhibitor cycloheximide was added at the time of chase. Actinomycin was added 30 min earlier. Data are derived from an electrophoretic analysis of whole cells and nuclei on polyacrylamide gels, using methods described in Taylor et al. (1969).

An investigation has also been made of the energy requirements of nuclear accumulation. The following metabolic inhibitors were used at the concentrations recommended by Jamieson and Palade (1968) in their investigations on the intracellular transport of secretory proteins in pancreatic slices: sodium fluoride (0.01 M), potassium cyanide (0.001 M) and 2,4-dinitrophenol (0.001 M). None had a demonstrable effect on nuclear accumulation when added at the beginning of the chase period.

Distribution of Viral Polypeptides between Nucleoplasm and Nucleoli

The following experiment was designed to examine the distribution of VP2 and VP3 within the nuclei. A suspension culture of HeLa cells, infected at a multiplicity of 10^2 ID_{50}/cell, was labeled from 3 to 6 hours after infection with ^{14}C-amino acids. Nuclei were extracted from the cells as previously described, then fractionated further into "nucleolus" and "nucleoplasm" by the method of Penman et al. (1967). Samples of each fraction were dissociated and analyzed on polyacrylamide gels, with results as shown in Fig. 3. It can be clearly
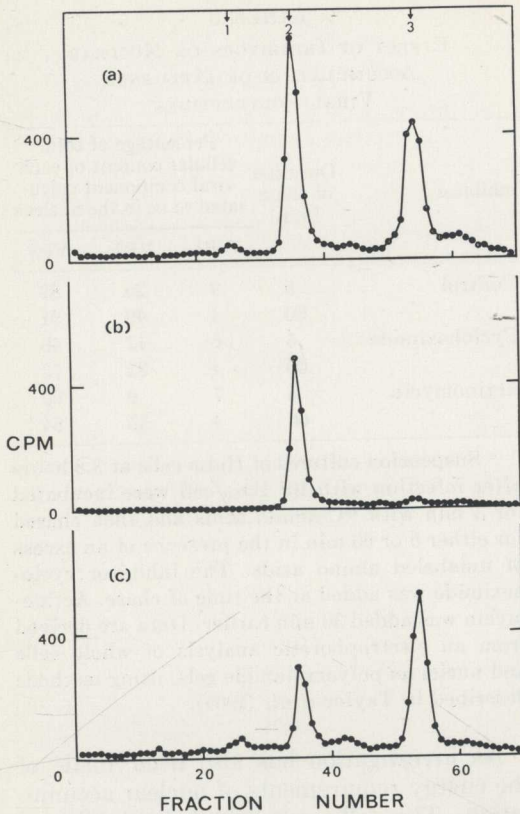
FIG. 3. Distribution of viral polypeptides between nucleolus and nucleoplasm of infected HeLa cells. Infected cells were labeled and fractionated as described in the legend of Fig. 1. Isolated nuclei were then fractionated by the method of Penman *et al.* (1967), to yield nucleoli and nucleoplasm, which were then dissociated and analyzed by electrophoresis as in Fig. 1: (a) nuclei, (b) nucleoplasm, (c) nucleoli.

seen that the nucleoplasm contains almost exclusively VP2. In fact, the ratio VP2:VP3 is 23 times higher in the nucleoplasm than in the original nuclei. On the other hand, the nucleoli contain virtually all the VP3; the VP2:VP3 ratio is 2.7 times lower than in the nuclei.

Nuclear Accumulation of Polypeptides of Newcastle Disease Virus

One respect in which the paramyxoviruses are considered to differ from the myxoviruses is that the nucleus does not seem to play a key role in their multiplication. Hence, it was of considerable interest to determine whether any of the polypeptides
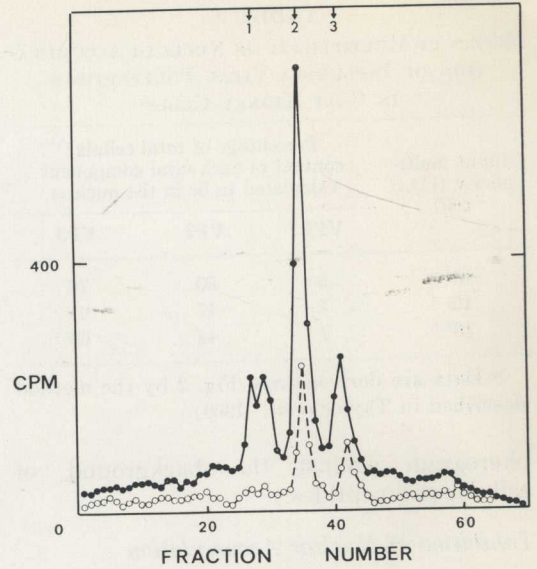

FIG. 4. Nuclear accumulation of viral polypeptides in HeLa cells infected by Newcastle disease virus. Experimental conditions were identical with those of Fig. 1, except that the culture was exposed to $10^{2.0}$ ID$_{50}$/cell of NDV. Solid line = whole cells; broken line = nuclei.

of a paramyxovirus accumulate in the nucleus of infected cells, and previous experience with Newcastle disease virus (NDV) made it a suitable choice. Like influenza, NDV contains three major polypeptides (Evans and Kingsbury, 1969; Haslam *et al.*, 1969), the slowest electrophoretic component, VP1, corresponding to the hemagglutinin, VP2 to the polypeptide of the nucleocapsid, and VP3 being unidentified.

A culture of HeLa cells was infected with NDV at a multiplicity of $10^{2.0}$ ID$_{50}$/cell. At 3.8 hours after infection, the cells were labeled for 10 min with ^{14}C-amino acids. Nuclei and whole cells were analyzed on polyacrylamide gels (Fig. 4). As in the case of influenza virus, VP2 and VP3 accumulate in the nucleus. The percentage of each of the three viral components calculated to be present in the nucleus after the 60 min chase was 12, 34, and 55%, for VP1, VP2, and VP3, respectively.

DISCUSSION

With HeLa cells infected at high multiplicity by influenza type A/Eel followed by

cell fractionation and polyacrylamide gel electrophoresis, it has previously been demonstrated that two of the three major structural polypeptides of the virus, VP2 and VP3, accumulate in the nucleus soon after their synthesis in the cytoplasm (Taylor et al., 1969). Another group using similar procedures has made a similar observation during the infection of chick embryo cells by a related myxovirus, fowl plague (Joss et al., 1969). One of the two nuclear polypeptides has been identified as that of the viral ribonucleoprotein, while the function of the other remains unknown (Haslam et al., 1970b). The investigations reported here represent an attempt to reveal the basis of nuclear accumulation.

Nuclear accumulation was not dependent on the multiplicity of infection within the range tested ($10^{0.5}$–$10^{2.0}$ ID$_{50}$/cell for an adsorption time of 30 min at room temperature). It is possible that the phenomenon would be less striking at m.o.i. of less than one, as appears to be the case with the nucleocapsid of measles virus studied by electron microscopy (Nakai et al., 1969).

Nuclear accumulation was observed not only during infection of HeLa cells, which do not support a productive cycle of infection by influenza virus (Henle et al., 1965), but also during the infection of primary embryonic calf kidney cells, which are fully permissive. These results do not prove that nuclear accumulation is essential for viral replication, but they do indicate that it is not an atypical event confined to abortive cycles of multiplication. Immunofluorescence studies on the intracellular localization of the "soluble antigen" have also indicated that the antigen accumulates in the nucleus during infection of permissive or nonpermissive cells (Fraser, 1967).

Certain metabolic inhibitors were examined to see whether they could inhibit the migration of the viral proteins from the cytoplasm into the nucleus. Migration was found to be unaffected by inhibition of protein synthesis with cycloheximide, DNA-dependent-RNA synthesis with actinomycin, or energy production with a variety of metabolic poisons. These findings suggest that the polypeptides enter the nucleus by simple diffusion, rather than by active transport. The time-dependent accumulation

of VP2 and VP3 in the nucleus, together with the fact that they cannot be washed out of the nucleus during the purification procedures, indicate that these viral polypeptides become bound inside the nucleus. On the other hand, the situation with VP1, the hemagglutinin, seems to be quite different, in that this molecule, which is a glycoprotein, becomes incorporated into plasma membranes (White et al., 1970).

One respect in which the paramyxoviruses differ from the myxoviruses is that they do not require a functional cell nucleus for their replication (Barry et al., 1962; Cheyne and White, 1969). Nevertheless, immunofluorescence studies report that the soluble antigen of certain paramyxoviruses may be found in the nucleus (Johnson and Scott, 1964; Llanes-Rodas and Liu, 1965). The results reported in this paper indicate that two of the three major structural polypeptides of NDV migrate into the nucleus. As with influenza virus, one of these (VP2) has been identified as the polypeptide of the viral nucleocapsid, while the function of the other (VP3) remains unknown (Haslam et al., 1969).

The intranuclear distribution of VP2 and VP3 in influenza-infected HeLa cells was examined, using the nuclear fractionation procedure of Penman et al. (1967). Most of the nucleocapsid polypeptide (VP2) was found in the nucleoplasm, while virtually all the VP3 was in the nucleolus.

Nucleoli fractions obtained by methods such as this really consist of a mesh of condensed ribonucleoprotein fibrils, a small proportion of which are not within the nucleolus, as defined morphologically (Penman et al., 1967; Georgiev, 1967). Nevertheless, the fact that the polypeptide of lower MW (VP3) is confined almost exclusively to the "nucleolus" whereas the higher MW VP2 is found predominantly in the "nucleoplasm" gives one confidence that the Penman separation is a meaningful one.

Although the present communication has shown that the accumulation of VP2 and VP3 in the nucleus occurs in a wide range of experimental situations, the relevance, if any, of this phenomenon to viral multiplication has yet to be established. However, since much of the VP2 and almost all of the VP3 enters the nucleus, it is tempting to suggest

that these "structural" proteins may play some additional role in viral replication.

The ribonucleoprotein polypeptide VP2, has been found in the nucleoplasm, and this is in agreement with studies employing immunofluorescence (Dimmock, 1969), ferritin-conjugated antibodies (Morgan *et al.*, 1961) and electron microscopy (Kopp *et al.*, 1968; Saito *et al.*, 1970). As the latter studies reveal RNP coils in the nucleus, it is likely that the VP2 is bound to viral RNA. The distribution of VP3 has not previously been studied, because this structural polypeptide of the virus has been demonstrated only recently (Pons and Hirst, 1969; Taylor *et al.*, 1969; Joss *et al.*, 1969; Holland and Kiehn, 1970) and even more recently shown to represent a new structural protein of the virion, distinct from those of the hemagglutinin, nucleocapsid and neuraminidase (Haslam *et al.*, 1970b). It may be relevant in this connection that Dimmock (1969) has detected a "nonstructural" antigen in the nucleolus of infected cells by immunofluorescence with mouse hyperimmune peritoneal fluid which had been absorbed with ether-disrupted virus. The findings reported in the present paper reveal only two species of polypeptide, identifiable as the structural polypeptides VP2 and VP3, in the nucleoli of influenza-infected cells. White *et al.* (1970) have postulated that VP3 occurs in the virion as a layer situated immediately beneath the peplomers and peripheral to the nucleocapsid. Such a protein may not have been available in antigenic form in the preparations of ether-disrupted virus used by Dimmock to absorb antibodies directed against structural proteins of the virion.

The function of VP3 however remains unproved. It is clearly a major structural protein of the virion, but it may also play an important role in viral replication. The observed rapid accumulation of this relatively small (MW 21,000) arginine-rich polypeptide in the nucleolus, which is a site at which transcription by a DNA-dependent RNA polymerase occurs (Roeder and Rutter, 1970) suggests two possible roles for VP3. Such a protein might represent part of the viral RNA-dependent RNA polymerase, or it might act as an inhibitor of cellular RNA synthesis. Indeed, the two possibilities are not necessarily mutually exclusive. Certain bacteriophage proteins of low MW have been shown to bind to the "core" of bacterial DNA-dependent RNA polymerase, so changing the template specificity from cellular to viral DNA (Bautz and Dunn, 1969). It is conceivable that VP3 may be acting in a similar way to redirect a cellular polymerase core from transcription of cellular DNA to the replication of viral RNA. Second, Skehel and Burke (1969) have partially purified viral polymerase activity from cells infected by fowl plague virus, and found that only two major viral proteins are associated: the protein of the nucleocapsid and a protein of MW 20,000, which is almost certainly VP3.

It is important to note that the site of synthesis of influenza viral RNA has yet to be established (Robinson and Duesberg, 1968). Studies of the intracellular distribution of viral polymerase have suggested that the activity is predominantly extranuclear (Scholtissek and Rott, 1969; Skehel and Burke, 1969). In interpreting these results, it should be recalled that application of aqueous cell fractionation procedures of a similar nature has sometimes erroneously indicated that most of the cell's DNA-dependent DNA polymerase is located in the cytoplasm (Keir *et al.*, 1962).

The following hypothesis is advanced to describe the events in the multiplication of influenza virus which take place following attachment and uncoating of the virion. (1) VP3, derived from the infecting virion or by direct translation of its RNA, binds to cellular DNA-dependent RNA polymerase, and so alters the specificity of the enzyme, enabling it to transcribe viral RNA. (2) Replication of the viral RNA takes place in the nucleus. (3) Newly synthesized viral RNA migrates to the cytoplasm where it is translated on polyribosomes. (4) Newly synthesized VP3 diffuses rapidly into the nucleolus and binds to the remaining cellular RNA polymerase, thus converting it to influenza virus-specific RNA polymerase and, at the same time, inhibiting synthesis of cellular messenger RNA. (5) Newly synthesized VP2 diffuses into the nucleoplasm, where it binds to viral RNA forming nucleocapsids; this association may also take place

to some extent in the cytoplasm. (6) Newly synthesized VP1 is converted to hemagglutinin by the attachment of carbohydrate, polymerization and eventual incorporation into plasma membranes. (7) VP2a, a minor structural polypeptide (Haslam *et al.*, 1970b), also incorporates carbohydrate and polymerizes to become the viral neuraminidase, embedded in plasma membranes. (8) Nucleocapsids, either from the cytoplasm or after migration from the nucleus, align themselves against modified plasma membrane; virions are then formed by budding.

Certain aspects of this model are highly speculative. They are put forward solely because they seem to be amenable to experimental test.

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The Polypeptides of Influenza Virus and Their Biosynthesis

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Influenza virus contains at least three functionally distinct proteins: the haemagglutinin and the neuraminidase, which are located in the envelope of the virion, and the protein of the internal nucleocapsid. All three of these proteins are clearly different, since they are antigenically distinct (Webster *et al.*, 1968), individually inherited in genetic crosses (Laver and Kilbourne, 1966), and physically separable by a variety of techniques (Laver, 1963, 1964; Laver and Valentine, 1969; Drzeniek *et al.*, 1966; Noll *et al.*, 1962; de Thé and O'Connor, 1966).

The synthesis of these viral proteins has been studied in several cell systems, usually by monitoring the appearance of functional viral proteins, or detecting their presence in an antigenically active form by immunofluorescence (Schäfer, 1959; Hillis *et al.*, 1960; Fraser, 1967; Rott and Scholtissek, 1967). It has been shown repeatedly that the 'soluble' (S, or G) antigen of the nucleocapsid is first detected in the nucleus of infected cells, while the 'viral' (V) antigen, considered in the past to correspond to viral haemagglutinin, is found only in the cytoplasm. More recently, Becht (1969) has presented autoradiographic data demonstrating increased incorporation of arginine into the nuclei of infected cells. From such results it has been suggested that one or all of the viral proteins may be synthesised in the cell nucleus.

The present paper describes studies on influenza virus proteins the level of their individual polypeptide chains. The main objectives of the work were to establish: (1) the identity and functional role of each polypeptide found in the virion; (2) the site of synthesis of these polypeptides, and their subsequent movements within the infected cell.

The Polypeptides of the Influenza Virion

The A0/Bel strain of influenza was grown in primary embryonic calf kidney cell monolayers from a small inoculum in order to minimize the

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production of incomplete virus. Proteins were labelled by incorporating ^{14}C-amino acids into the maintenance medium at the time of infection. The virus released from the cells was extensively purified by a procedure involving both rate zonal and equilibrium gradient centrifugation, described in Table I.

Table I. Purification of Influenza Virus

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1. Virus grown in primary calf kidney cell monolayers.
2. Medium clarified by a low-speed centrifugation.
3. Virus pelleted onto a 'cushion' of 2·3M sucrose.
4. Virus centrifuged to a central position on a rate-zonal gradient of 15–60% sucrose (w/v).
5. Virus centrifuged to density equilibrium on a gradient of 20–40% potassium tartrate (w/v).

The purified virus was dissociated and analysed by polyacrylamide gel electrophoresis using the neutral sodium dodecyl sulphate (SDS) system of Maizel (1969). First, the virus was broken down to its constituent polypeptides using SDS in the presence of 2-hydroxyethyl disulphide and catalytic quantities of 2-mercaptoethanol. The resulting polypeptides have lost all secondary and tertiary structure and bind enough SDS to give them all a net negative charge, so that they can be resolved by electrophoresis at neutral pH in SDS-containing acrylamide gels according to their molecular weights (Maizel, 1969; Shapiro and Maizel, 1969). When influenza virus was analysed in this way, three major viral polypeptides (VP1, 2, 3) were resolved (Fig. 1). On occasions, VP1 appeared to consist of two components. Other minor components were sometimes seen.

For comparison, unlabelled influenza virus was grown in the allantois of chick embryos, or in calf kidney cell cultures, then purified, dissociated and analysed in the same way. The resulting bands of protein were stained with Coomassie blue (Maizel, 1969). The bands coincided in number and position with those obtained by counting of radioactive virus, indicating that all the major polypeptides of the influenza virion are newly synthesized. Therefore, the virion contains no significant amount of any particular preformed host protein, although the presence of trace amounts of several different host proteins cannot be completely excluded. When these gels were also stained with the periodic acid–Schiff reagent, both components of VP1 consistently stained bright red, indicating the presence of carbohydrate. It may be concluded that this material consists of glycopeptide.

The molecular weights of the viral polypeptides were estimated by

measuring their migration in polyacrylamide gels in comparison with that of SDS-treated proteins of known molecular weight. A linear calibration curve was constructed by plotting the distance migrated by each marker protein against the logarithm of its molecular weight (Shapiro and Maizel, 1969; Weber and Osborn, 1969); the molecular
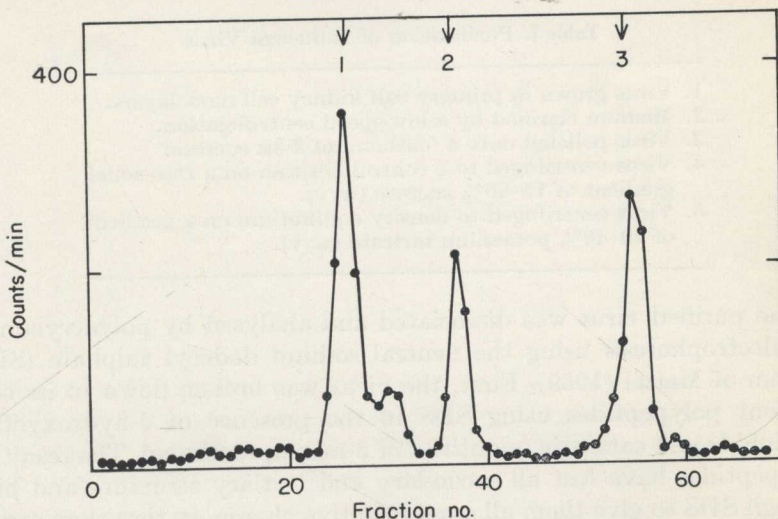

Fig. 1. Polypeptide composition of influenza virus. The A_0/Bel strain of influenza virus was grown for 72 h in primary embryonic calf kidney cells (multiplicity of infection $= 0.05$ tissue culture ID_{50}/cell) in the presence of ^{14}C-amino acids. After purification, the virus was dissociated with 2% sodium dodecyl sulphate (SDS), 0·5M urea, 0·04M 2-mercaptoethanol and 0·1M 2-hydroxyethyl disulphide in 0·01M phosphate buffer, pH 7·2. The mixture was incubated at 37°C for 30 min, then 100°C for 1 min, and dialysed against 0·1% SDS and 0·5M urea at pH 7·2. The resultant polypeptides were separated by electrophoresis at pH 7·2 in 5% polyacrylamide gels (0·6 × 20 cm) containing 0·1% SDS and 0·5M urea. After 19·2 hours at 50 volts the gels were fractionated and counted. Migration in this and all subsequent diagrams is towards the anode on the right (reproduced from Taylor *et al.*, 1969).

weight of each viral polypeptide was then deduced from its own migration (Table II).

It is possible that the MW given for VP1 is an over-estimate for the polypeptide itself because it is linked to an unknown amount of carbohydrate, and the migration of glycoproteins in the neutral SDS–acrylamide gel system has not yet been tested for any species containing more than about 6% carbohydrate (Weber and Osborn, 1969). A glycoprotein containing a large amount of carbohydrate might be expected to bind less SDS than protein of comparable MW and hence migrate less rapidly towards the anode. The table also gives the contribution of each of the three major polypeptides to the total virion

Table II. The Molecular Weights and Relative Proportions of Influenza Viral Polypeptides

VP	Molecular weight[a]	Percentage of virion protein[b]	Molecular ratio per virion[c]	Number of molecules per virion[d]
1	74000[e]	38	1·0[e]	1000[e]
2	50000	17	0·7	700
3	20000	27	2·7	2700

[a] Estimated by the method of Shapiro and Maizel (1969).

[b] From Fig. 1; the values given are the mean of several determinations. The counts in each peak were expressed as a percentage of the total counts recovered from the gels.

[c] Calculated by dividing the percentage of virion protein by the estimated molecular weight, and normalizing the resultant ratios with respect to peak 1 as unity.

[d] Calculated on the assumption that the mass of an average influenza virion is approximately 3×10^8 daltons, and that about 70% of this is protein (Hoyle, 1968).

[e] The carbohydrate moiety of VP1 might reduce its electrophoretic migration in this system. Hence, the MW of 74,000 obtained by the Shapiro–Maizel method may be an over-estimate. In this event, the figures in the two right-hand columns would be under-estimates.

protein, expressed firstly as a percentage of the total mass of viral protein and secondly on a molecular basis.

Identification of the Polypeptides

An attempt was made to correlate the main polypeptides of influenza with the known structural proteins of the virion. The basic approach was to isolate sub-viral components with recognizable biological activity, then dissociate them to their component polypeptides for analysis by electrophoresis. The dilemma in this kind of work is that the smaller the structure the greater the probability that it contains a single protein species but the less the chance that it retains its biological activity.

Purified, radioactively labelled influenza virus was disrupted with sodium deoxycholate. After centrifugation at 95,000 **g** for 60 minutes, the pellet and the supernatant fluid were assayed for haemagglutinating and neuraminidase activities, then dissociated and analysed by gel electrophoresis (Fig. 2).

VP1 and VP3 were found mainly in the supernatant fraction, whereas the pellet was relatively enriched in VP2. It was considered that VP1 and VP3, being readily detached from the virion, are probably relatively superficial proteins, while VP2 is situated internally. Furthermore,
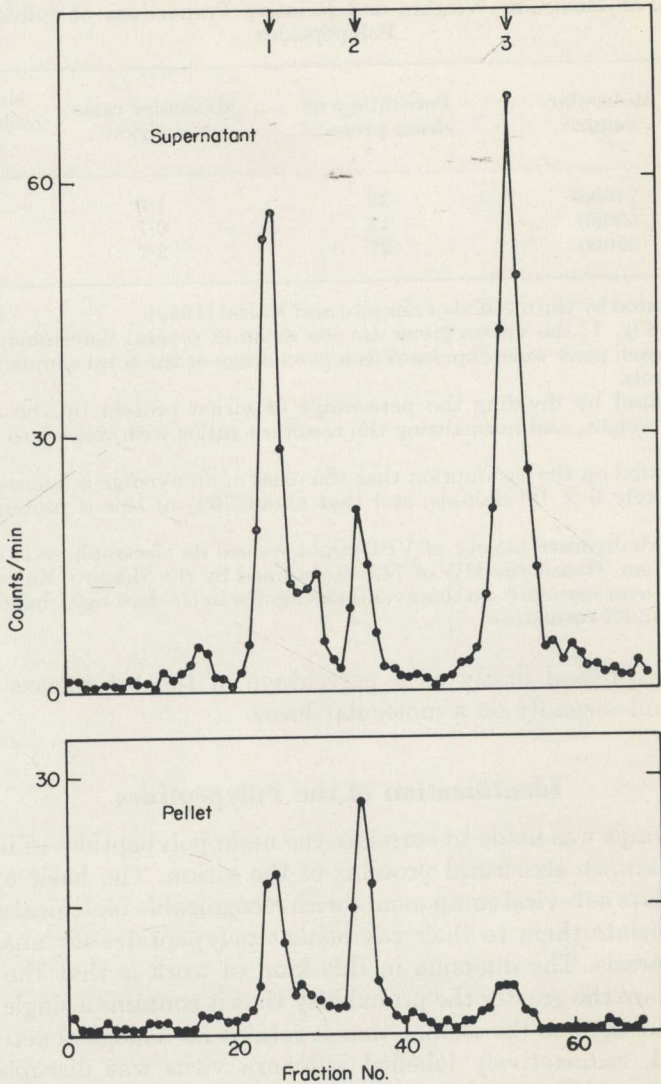
Fig. 2. Polypeptide composition of fractions from deoxycholate-disrupted influenza virus. Purified influenza virus was stirred for 1 h at 4°C in the presence of 1% sodium deoxycholate at pH 8·0. The mixture was then centrifuged at 95,000 g for 60 min. The pellet was resuspended by ultrasonic vibration. Both pellet and supernatant fluid were assayed for haemagglutinin and neuraminidase activities, then dissociated and analysed by polyacrylamide gel electrophoresis as described in Fig. 1. In this and all subsequent electrophoretograms the migration of the three major viral polypeptides, as determined with marker virus, is indicated by numbered arrows.

since the supernatant fraction contained more than half of the residual neuraminidase and haemagglutinating activity (and probably most of the additional neuraminidase and haemagglutinin that had been inactivated by deoxycholate), the experiment suggested that VP1 and VP3 may be concerned with these two functions, whereas VP2 might comprise the ribonucleoprotein. Nevertheless, definitive identification
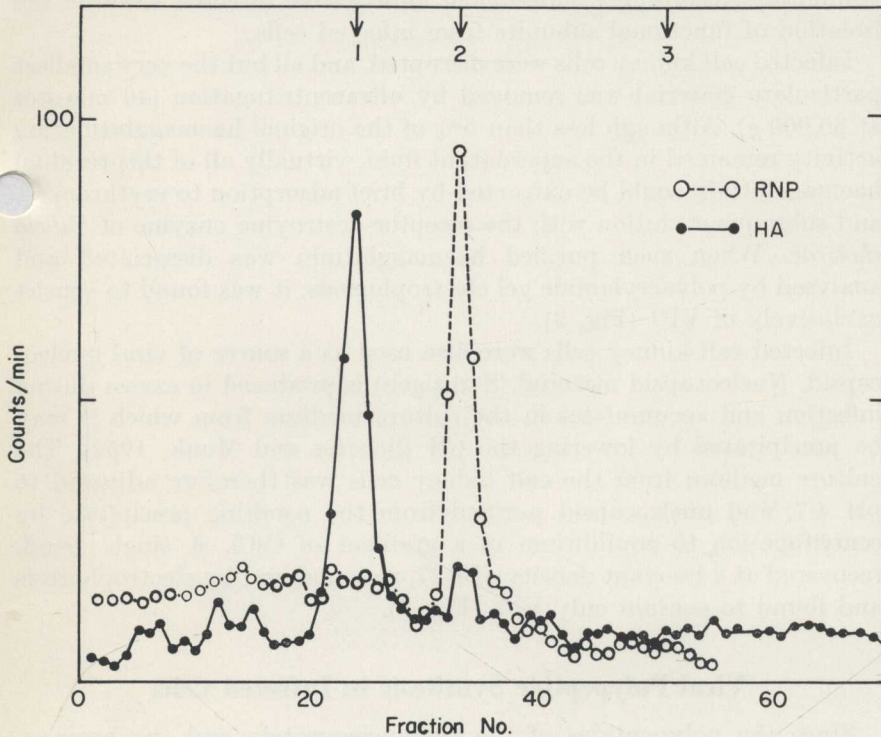

Fig. 3. Identification of haemagglutinin and nucleocapsid polypeptides of influenza virus. Haemagglutinin was isolated from virus-infected calf kidney cells as follows. The cells were disrupted by ultrasonic vibration; cell debris, virions and large subviral particles were removed by centrifugation for 40 min at 80,000 *g*, and the supernatant fluid was treated for 5 min at 0°C with fowl erythrocytes at a final concentration of 3%. The erythrocytes were collected by centrifugation and the adsorbed haemagglutinin was eluted by incubation at 37°C for 1 h (at pH 6·2 in the presence of calcium) with neuraminidase from *Vibrio cholerae*.

Nucleocapsid protein was isolated from the calf kidney culture medium as follows. The medium was centrifuged at 64,000 *g* for 30 min to remove virus particles, and the pH of the supernatant fluid was adjusted to 4·7. The precipitate, which formed overnight at 4°C, was resuspended and layered onto a gradient of 11–38% (w/w) CsCl. The gradient was centrifuged for 15 h at 180,000 *g* in an SW65 Spinco rotor, and the single band on the gradient (buoyant density 1·27) was harvested.

The haemagglutinin and the nucleocapsid protein were dissociated and analysed by polyacrylamide gel electrophoresis as described in Fig. 1.

of the polypeptides required more rigorous separation of functional subunits. Disruption of labelled virions was therefore attempted using several other methods, including Tween 80 plus ether, previously used in this laboratory for the identification of NDV polypeptides (Haslam et al., 1969). Such methods confirmed VP2 as an internal protein, but failed to separate active haemagglutinin from neuraminidase. Accordingly, subsequent efforts were directed towards the isolation of functional subunits from infected cells.

Infected calf kidney cells were disrupted, and all but the very smallest particulate material was removed by ultracentrifugation (40 minutes at 80,000 g). Although less than 5% of the original haemagglutinating activity remained in the supernatant fluid, virtually all of this residual haemagglutinin could be extracted by brief adsorption to erythrocyte and subsequent elution with the receptor-destroying enzyme of *Vibrio cholerae*. When such purified haemagglutinin was dissociated and analysed by polyacrylamide gel electrophoresis, it was found to consist exclusively of VP1 (Fig. 3).

Infected calf kidney cells were also used as a source of viral nucleocapsid. Nucleocapsid material (S antigen) is produced in excess during infection and accumulates in the culture medium from which it may be precipitated by lowering the pH (Schäfer and Munk, 1952). The culture medium from the calf kidney cells was therefore adjusted to pH 4·7, and nucleocapsid purified from the resulting precipitate by centrifugation to equilibrium in a gradient of CsCl. A single band, recovered at a buoyant density of 1·27, was analysed by electrophoresis and found to contain only VP2 (Fig. 3).

Viral Polypeptide Synthesis in Infected Cells

Since the polypeptides of the ribonucleoprotein and the haemagglutinin could be readily identified by gel electrophoresis, it was practicable to re-examine the details of the intracellular synthesis an migration of these proteins. Two cell systems were used: suspension cultures of HeLa cells and monolayer cultures of primary embryonic calf kidney cells. The suspension cultures are convenient for biochemical experiments requiring rapid manipulation of large numbers of cells but suffer from the disadvantage that the virus undergoes an abortive cycle of multiplication not leading to any significant production of infectious virus (Henle et al., 1955). For both systems it was considered necessary to infect at high multiplicity of infection in order to infect all the cells and to synchronize the events of the viral multiplication cycle. The synthesis of the viral polypeptides was therefore studied under

conditions which usually result in the production of incomplete virus (von Magnus, 1954), even in permissive cell lines.

The time course of infection of HeLa and calf kidney cells by high multiplicities of influenza virus was analysed. The results for the two cell systems were similar; those obtained with HeLa cells are presented
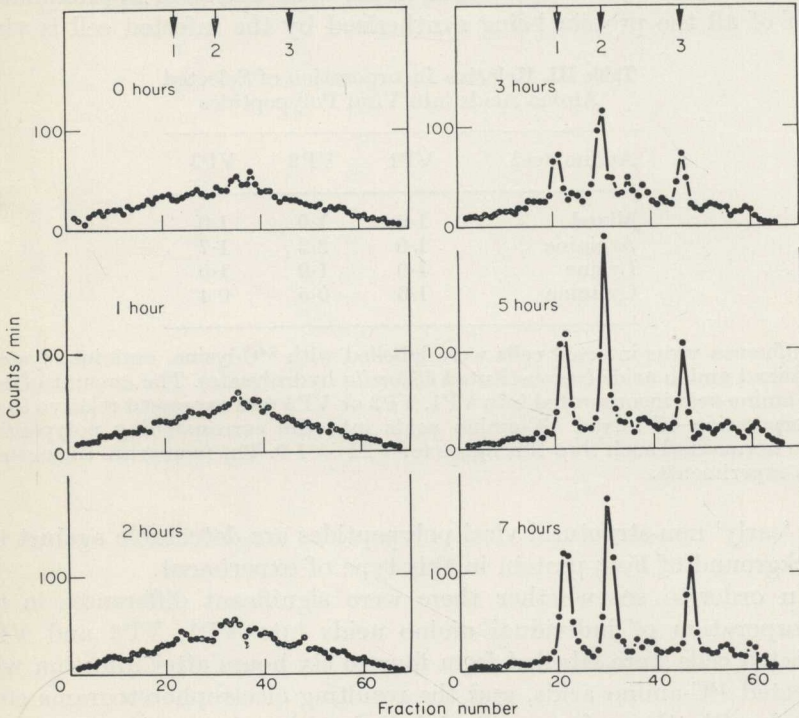

Fig. 4. Polypeptides synthesized by HeLa cells infected with influenza virus. HeLa cells from suspension culture were washed and resuspended at a concentration of 10^7 cells per ml. Influenza virus was added at high multiplicity and allowed to adsorb for 30 min at room temperature. The cell suspension was then diluted 10-fold with warm amino acid-free medium and incubation continued at 37°C. At hourly intervals after infection, aliquots of cells were removed and labelled for 15 min with ^{14}C-amino acids. Samples were dissociated and analysed by polyacrylamide gel electrophoresis as described in Fig. 1. The electrophoretograms have been normalized to represent equal total radio-activity.

in Fig. 4. From three hours after infection the cells are clearly synthesizing large amounts of three polypeptides, which migrate in polyacrylamide gel electrophoresis identically with the three major structural polypeptides seen in purified virus grown in calf kidney cells (Fig. 1). The synthesis of all three of the main viral polypeptides increases rapidly between two hours and four hours after infection.

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Throughout the cycle these three viral polypeptides are synthesized in constant proportions relative to one another. However, the ratio between them does not correspond with that seen in the virion itself; the ribonucleoprotein (VP2) is present in relative excess in the infected cell. Host protein synthesis declines in parallel with the increase in viral structural protein; by four hours after infection approximately half of all the protein being synthesized by the infected cell is viral.

Table III. Relative Incorporation of Selected
Amino Acids into Viral Polypeptides

Amino acid	VP1	VP2	VP3
Mixed	1·0	1·0	1·0
Arginine	1·0	2·2	1·7
Lysine	1·0	1·0	1·0
Cysteine	1·0	0·5	0·4

Influenza virus-infected cells were labelled with ^{14}C-lysine, arginine, cysteine or mixed amino acids (reconstituted *Chlorella* hydrolysate). The amount of each ^{14}C-amino acid incorporated into VP1, VP2 or VP3 was expressed relative to the incorporation of mixed ^{14}C-amino acids into the corresponding polypeptide, then normalized such that this figure for VP1 = 1·0. The figures are the mean of two experiments.

No 'early' non-structural viral polypeptides are detectable against the background of host protein in this type of experiment.

In order to see whether there were significant differences in the incorporation of individual amino acids into VP1, VP2 and VP3, infected cells were labelled from four to six hours after infection with selected ^{14}C-amino acids, and the resulting electrophoretograms compared with those from cells labelled with mixed ^{14}C-amino acids (Table III).

VP1 differs strikingly from VP2 and VP3 in its content of arginine and cysteine. Hence, clearly neither VP2 nor VP3 constitutes a poly-peptide precursor of the glycopeptide, VP1. Further implications of these differences will be discussed below.

Cytoplasmic Synthesis and Nuclear Accumulation

For some years there has been controversy in the literature about the intracellular site of synthesis of influenza proteins and RNA (see Robinson and Duesberg, 1968). This question has recently been re-investigated in our laboratory, using a short 'pulse-chase' followed by cell fractionation and electrophoresis (Taylor *et al.*, 1969). HeLa cells

were infected with influenza virus, and at 3·8 hours were labelled for
three minutes with ^{14}C-amino acids, then 'chased' in the presence of
unlabelled amino acids for either one minute (to allow time for the
completion of most of the polypeptides still nascent on polyribosomes)
or 60 minutes. Nuclei were extracted using a Dounce homogenizer,
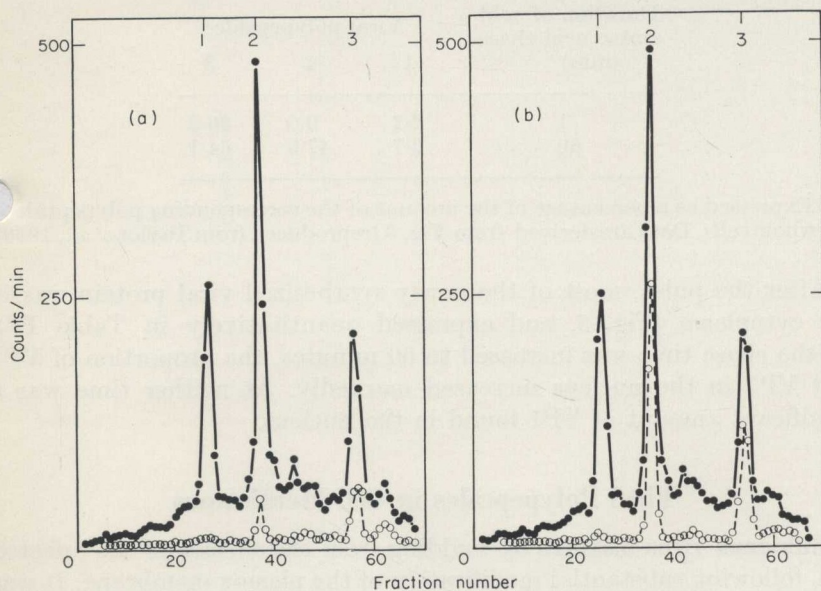washed with Nonidet P40, and centrifuged through sucrose. Samples

Fig. 5. Cytoplasmic synthesis and nuclear accumulation of viral polypeptides in HeLa
cells infected with influenza virus. At 3·8 h after infection the cells were washed, resus-
pended in amino acid-free medium, and labelled for 3 min with ^{14}C-amino acids, then
chased in the presence of unlabelled amino acids for (a) 1 min, or (b) 60 min. To extract
the nuclei, the cells were swollen in hypotonic buffer and disrupted in a Dounce homo-
genizer. Further purification of the nuclei was achieved by washing with the non-ionic
detergent Nonidet P40 and centrifuging through 2·3M sucrose (Taylor et al., 1969). These
nuclear preparations, together with samples of the original infected cells, were dissociated
and analysed by polyacrylamide gel electrophoresis. In each electrophorogram the
solid line represents whole cells, and the broken line, the nuclei. The data has been
normalized to represent an equivalent number of whole cells or nuclei (reproduced from
Taylor et al., 1969).

of both nuclei and whole cells were analysed by polyacrylamide gel
electrophoresis (Fig. 5).

The effectiveness of the chase was demonstrated by the fact that the
acid-precipitable radioactivity did not increase when the chase was
extended from one to 60 minutes. Furthermore, examination of the
electrophorograms of whole cells reveals that the radioactivity in VP1,
VP2 and VP3 did not alter during the chase. In other words, all three

viral polypeptides are stable; none is a short-lived precursor of the type recognized with some of the picornaviruses and arboviruses (Maizel and Summers, 1968.)

Table IV. Percentage of each Viral Polypeptide found in the Nucleus[a]

Duration of cold amino acid chase (min)	Viral polypeptide		
	1	2	3
1	2·2	9·0	36·6
60	2·7	47·0	64·1

[a] Expressed as a percentage of the amount of the corresponding polypeptide the whole cells. Data are derived from Fig. 5 (reproduced from Taylor *et al.*, 1969).

After the pulse, most of the newly synthesized viral protein was in the cytoplasm (Fig. 5, and expressed quantitatively in Table IV). As the chase time was increased to 60 minutes, the proportion of VP2 and VP3 in the nucleus increased markedly. At neither time was a significant amount of VP1 found in the nucleus.

Viral Polypeptides in Cell Membranes

Influenza virus matures by budding from the surface of the infected cell, following substantial modification of the plasma membrane. It was felt that close investigation of the incorporation of viral polypeptides into the plasma membranes may throw light, not only on the morphogenesis of enveloped viruses, but also on the structure of the membranes themselves.

Influenza virus-infected HeLa cells develop the capacity to adsorb erythrocytes even though the multiplication cycle is abortive (Henle *et al.*, 1955; White *et al.*, 1965). Accordingly, a smooth membrane fraction was isolated from a homogenate of infected HeLa cells using a modification of the method of Hays and Barland (1966). Membrane fragments were floated through discrete layers of sucrose using isopycnic centrifugation. Material was collected from the interface characteristic of plasma membranes and analysed by polyacrylamide gel electrophoresis (Fig. 6).

The results demonstrate that the membranes contain a relatively large amount of VP1, i.e. the polypeptide of the haemagglutinin. In fact the relative content of VP1, with respect to VP2 and VP3, in these membranes was almost 10-fold higher than that observed in whole cells.

Preliminary experiments have shown that sodium azide (0·3 M) stops the incorporation of VP1 into membranes, indicating that energy is required at some stage between the synthesis of VP1 and its incorporation into plasma membranes. Work in progress is aimed at
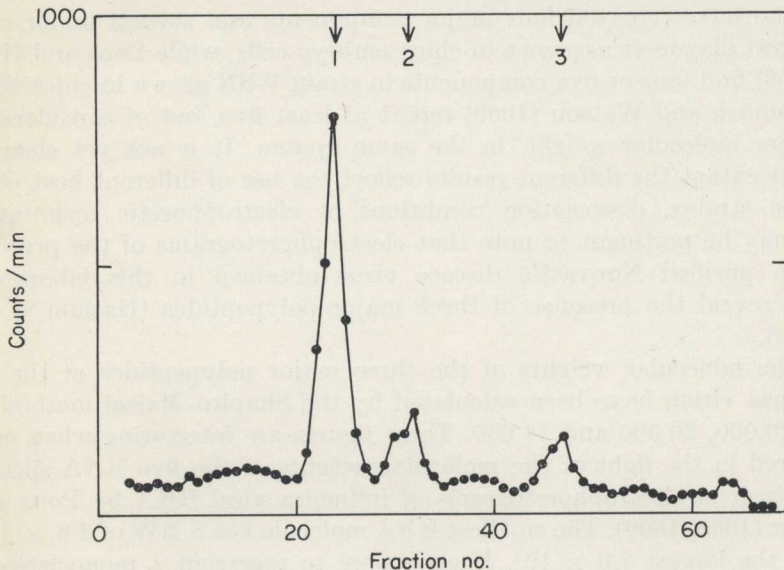

Fig. 6. Polypeptide composition of a membrane fraction of HeLa cells infected with influenza virus. Infected HeLa cells were labelled with ¹⁴C-amino acids from three to six hours after infection. The cells were then swollen in hypotonic buffer and disrupted with a Dounce homogenizer. The fraction containing plasma membranes was obtained from this homogenate by the following modification of the method of Hays and Barland (1966). The homogenate was centrifuged at 1500 g for 30 min; the resulting pellet was resuspended in 0·3 ml of 0·005M Tris at pH 7·4. This was mixed with 2 ml of 67% sucrose (w/w) in 0·005M Tris and successively overlaid with 3 ml volumes of 55%, 49% and 36% sucrose (w/w) in 0·005M Tris. After centrifugation to equilibrium (200,000 g for 45 min in an SW41 Spinco rotor), the 36–49% sucrose interface was collected as the membrane fraction. Following dilution, the membranes were centrifuged out of the sucrose (200,000 g, 20 min), dissociated and analysed by polyacrylamide gel electrophoresis as described in Fig. 1.

elucidating the sequence of events whereby this viral polypeptide becomes linked to carbohydrate, assembled into spikes and incorporated into the plasma membrane.

Discussion

It has been the experience of this laboratory (Haslam et al., 1970a, b; Taylor et al., 1969) that the purified virion of influenza A0/Bel contains

three major polypeptides. Duesberg and Robinson (1967) have previously reported the presence of three electrophoretic components in the protein of the PR8 strain grown in embryonated eggs. Griffith (1968) has also observed three bands in stained electrophoretograms of protein from a number of egg-grown strains. More recently, Skehel and Burke (1969) have reported four major components and several minor ones in fowl plague virus grown in chick embryo cells, while Pons and Hirst (1969) find four or five components in strain WSN grown in chick cells. Dimmock and Watson (1969) report at least five, but of considerably higher molecular weight, in the same system. It is not yet clear to what extent the different results reflect the use of different host cells, virus strains, dissociation conditions or electrophoretic techniques. It may be pertinent to note that electrophoretograms of the protein from purified Newcastle disease virus obtained in this laboratory also reveal the presence of three major polypeptides (Haslam et al., 1969).

The molecular weights of the three major polypeptides of the influenza virion have been calculated by the Shapiro–Maizel method to be 20,000, 50,000 and 74,000. These figures are interesting when considered in the light of the molecular weights of the five RNA species resolved in electrophoretograms of influenza viral RNA by Pons and Hirst (1968, 1969). The smallest RNA molecule has a MW of $2 \cdot 6 \times 10^5$, and the largest $7 \cdot 0 \times 10^5$. If each were to represent a monocistronic messenger, these five messenger RNA molecules would code for polypeptides ranging in MW from about 26,000 to 70,000. Assuming a MW of $2 \cdot 8 \times 10^6$ for the average complement of RNA per influenza virion (Robinson and Duesberg, 1968), approximately half of the influenza genome would be required to code for these three polypeptides alone.

The structural role of only two of the three main polypeptides of the virion has been identified with any certainty. VP1 is a glycoprotein component of the viral haemagglutinin. It is approximately twice as rich in cysteine as VP2 and VP3; the tertiary structure of the haemagglutinin spike may therefore be dependent on interchain or intrachain disulphide linkages. The presence of carbohydrate in VP1 may explain some of the differences between electrophoretic patterns of influenza virus proteins reported from various laboratories. Laver and Webster (1966) demonstrated that the carbohydrate moiety of the isolated haemagglutinin of A0/Bel had the antigenic specificity of the host cell. Hence, the structure and consequent electrophoretic mobility of the haemagglutinin glycoprotein VP1 could vary with both the virus strain and the host cell. VP2 corresponds to the nucleocapsid protein. It has a MW of 50,000 and is twice as rich in arginine as VP1. This was

demonstrated for influenza viral ribonucleoprotein many years ago (Hoyle and Davies, 1961) and is compatible with the association of this protein with RNA. It is not certain whether the high arginine content of VP3 also reflects an association with acidic groups in the virion. VP3, of MW 20,000, is the most plentiful polypeptide in the virion on a molecular basis, yet its structural role has still to be elucidated. The fact that it is relatively easily removed from the virion by deoxycholate suggests that it is not buried deeply within the particle. It is possible that it comprises a layer of protein, external to the nucleocapsid, which may stabilize the virion and serve as a point of attachment for the nucleocapsid or the base of the projections embedded in the viral envelope. In this connection it is interesting to note the 'granular layer' electron-dense material situated directly beneath the peplomers of the virion in the recent micrographs of Kendal et al. (1969). The polypeptide(s) of the neuraminidase has yet to be identified. It is difficult to obtain active enzyme molecules free of other components of A0/Bel. Preliminary results with the influenza B strain Lee, suggest that the monomer of the neuraminidase may correspond to a fourth, and relatively minor, polypeptide (Haslam et al., 1970b).

Several papers in the literature have demonstrated that influenza G(S) antigen first becomes detectable by immunofluorescence in the cell nucleus and only later passes to the cytoplasm, whereas V antigen (? haemagglutinin) is found exclusively in the cytoplasm (Schäfer, 1959; Hillis et al., 1960; Fraser, 1967). Furthermore, Becht (1969) has demonstrated by autoradiography the rapid incorporation of arginine into the nucleus of influenza-infected cells. The results reported in this paper are not incompatible with any of these data. What must be disputed is the conclusion that viral polypeptides are synthesized in the nucleus (see Rott and Scholtissek (1967) for discussion).

The experiments reported here strongly suggest that all three viral polypeptides are synthesized in the cytoplasm and that two of them subsequently migrate into the nucleus. There is, however, tan alernative interpretation of the data that cannot be completely excluded: (a) viral polypeptides may be synthesized in the nucleus, and (b) may leak out of the nucleus during the cell fractionation procedures, but (c) polypeptides 2 and 3 may not leak out if they have become incorporated in a time-dependent manner into some nuclear structure (e.g. nucleocapsid). While this interpretation cannot be formally disproved, essentially the same findings have been obtained using a variety of other methods of cell fractionation which do not yield such clean nuclei but subject them to such mild conditions (isotonic medium, low g-forces) that no loss of protein would be expected.

VP3, which has a MW of 20,000, moves into the nucleus considerably more rapidly than the nucleocapsid polypeptide, VP2, of MW 50,000 (Tables II and IV). This suggests that the migration may occur by a process of simple diffusion, at a rate which is inversely proportional to MW. Preliminary experiments have revealed that the migration is hardly affected by cycloheximide, actinomycin D, or sodium azide (Taylor, et al., in preparation). Because the three main structural polypeptides of influenza virus can be so readily followed around the cell by a combination of cell fractionation and gel electrophoresis, this may provide a useful system for studying the factors influencing the movement within cells of proteins in general.

The biological significance of this migration of the arginine-rich viral polypeptides into the nucleus remains to be established. Perhaps VP or VP3 serves as a late 'cell shutoff' protein, which binds directly to cellular nucleic acid. Alternatively, it could be envisaged that, for some reason, the viral nucleocapsid has to be assembled in the nucleus, perhaps utilizing facilities present there for the assembly of ribosomes from RNA and protein. It is known that influenza virus, unlike parainfluenza or NDV, cannot multiply in enucleated cells (Cheyne and White, 1969). However, it is important to stress that this does not mean that any crucial step in the transcription, translation or replication of influenza viral RNA, nor even the assembly of viral components, must necessarily take place in the nucleus; the finding may simply reflect a requirement for the synthesis of a particular cellular messenger RNA(s) during the first two hours of the multiplication cycle (Barry et al., 1962; White et al., 1965; White and Cheyne, 1966). It is entirely possible that the migration of viral polypeptides from the cytoplasm into the nucleus is of no crucial relevance to the processes of viral multiplication.

Interest in this laboratory is now being focused on the factors influencing the incorporation of viral polypeptides into membranes. Several important questions present themselves. One concerns the mechanisms involved in the linkage of carbohydrate to VP1 to form the glycopeptide of the haemagglutinin. For example, it is not known whether this occurs on polyribosomes, in the Golgi apparatus or in the plasma membrane itself. Evidence is accumulating which suggests that the carbohydrate component of glycoproteins is required for their transport through membranes. A systematic study of the incorporation of viral peplomers into plasma membrane may shed considerable light on the structure of the membrane itself.

Summary

Influenza virus has been extensively purified, dissociated into its component polypeptides and analysed by polyacrylamide gel electrophoresis. The virion contains three major polypeptides and probably some minor ones. By separating small subunits from radioactively labelled virions or infected cells it has been possible to identify the structural role of two of these polypeptides. The most slowly migrating electrophoretic component, a glycoprotein relatively rich in cysteine, is the fundamental subunit of the haemagglutinin. A relatively arginine-rich polypeptide of MW 50,000 corresponds to the ribonucleoprotein. Another arginine-rich polypeptide of MW 20,000 constitutes the most common molecule in the virion. Its precise structural role has yet to be determined, but it is situated outside the nucleocapsid and may stabilize the virion by serving as a point of anchorage for the peplomers and/or ribonucleoprotein. The MWs of these three polypeptides are consistent with the hypothesis that the several molecules of viral RNA represent monocistronic messengers. The polypeptide(s) comprising the neuraminidase has yet to be identified with certainty. No preformed host protein is detectable in the highly purified virion.

In HeLa or calf kidney cells all three major viral polypeptides are present by two hours after infection, and being synthesized at maximum rate by four hours. Throughout the cycle the three are made in a constant ratio which does not, however, correspond to that found in the virion itself; the ribonucleoprotein is synthesized in excess. All the viral polypeptides are stable; there is no evidence for a short-lived, high MW precursor. Host protein synthesis begins to decline only after viral structural proteins begin to be made; by four hours about half of all protein being synthesized by the infected cell is viral.

Cell fractionation following a brief pulse and chase indicates that all these viral polypeptides are synthesized in the cytoplasm. The polypeptide of the haemagglutinin becomes incorporated into membranes. The two arginine-rich polypeptides, on the other hand, move into the nucleus.

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The Polypeptides of Adenovirus

I. Evidence for Multiple Protein Components in the Virion and a Comparison of Types 2, 7A, and 12[1]

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The adenovirion has been shown to contain at least nine different polypeptides demonstrable by dissociation and acrylamide gel electrophoresis in the presence of sodium dodecyl sulfate (SDS). Each polypeptide of adenovirus type 2 is chemically distinct by isotopic ratio analysis, and they all contain lysine, arginine, tryptophan, valine, and threonine. The molecular weights of these polypeptides determined by gel electrophoresis range from 120,000 for the largest and most prominent component to 7500 for the smallest. Comparison of nontumorigenic type 2 with tumorigenic types 7A and 12 by double-isotope labeling revealed a generally similar peptide pattern for all types. However, there were distinct differences between the corresponding peptides of all three types. These results imply extensive differences in the genes for most of the capsid proteins.

INTRODUCTION

The human adenovirus group comprises more than 30 immunologically related but different types (Ginsberg, 1962; Pereira *et al.*, 1963). In addition to being infectious for man, some types (3, 7, 12, 18, and 31) are tumorigenic in newborn hamsters (Green, 1966). Cultured cells infected with adenoviruses contain, in addition to quantities of easily purified virions, large amounts of virus-related antigens that are not assembled into virions (Klemperer and Pereira, 1959; Philipson, 1960; Wilcox and Ginsberg, 1961). The electron microscopic appearance of both the virion and the soluble antigens

have been described in detail (Wilcox *et al.*, 1963; Valentine and Pereira, 1965). In order to better define the structure of this large (800 Å diameter), DNA-containing icosahedral virus, its polypeptide composition has been investigated. In a number of preliminary reports (Maizel, 1964, 1966; Maizel and Scharff, 1966; Maizel *et al.*, 1967), the virion was reported to contain at least nine distinct polypeptide chains separable by electrophoresis on acrylamide gels. The present paper will (1) describe methods for the disruption of the virion into its individual polypeptide chains, (2) catalog the polypeptide chains on the basis of their migration in SDS-containing acrylamide gels, (3) present evidence for the physical and chemical discreteness of most of the nine polypeptides, and (4) compare the electrophoretic patterns of types 2, 7A, and 12. In the following papers (Maizel *et al.*, 1968 this issue; White *et al.*, in preparation), the location of most of these polypeptide chains in the virion will be established and their synthesis in infected cells described.

METHODS

Cells and virus. Adenovirus types 2 and 12 stocks were grown from initial seed virus kindly provided by Dr. David Axelrod of the Laboratory of Biology of Viruses, NIH, Bethesda, Maryland, and did not differ from stocks obtained from the American Type Culture Collection. Inocula of types 7A and 12 (Huie) were also provided by the Viral Research Reference Collection of the National Institutes of Health. KB cells (Eagle, 1955), and a clonal line of HeLa S3-1 isolated by Dr. N. P. Salzman were grown in Eagle's medium (Eagle, 1959) supplemented with 5% calf serum.

Preparation of concentrated and purified virus stocks. Crude virus stocks were prepared by infecting stationary Blake bottle cultures containing 2×10^7 cells with 0.1 ml of seed virus. After 3 days, when the cell monolayers were completely detached from the glass, the entire culture was frozen and thawed three times and stored at 4°C ("stock" virus). To prepare larger amounts of virus for purification, 5 ml of the above stock was added to 600–700 ml of suspension culture at 1.5×10^5 cells/ml, and the infected culture incubated for 40–48 hours. The virus was then purified by a modification of the method of Green and Piña (1964).[4] The washed cells were extracted three times with Freon, and the aqueous phase was layered on a 20-ml linear CsCl gradient ranging in density from 1.2 to 1.4 g/ml, centrifuged for 3 hours at 25,000 rpm in a Spinco SW 25.1 rotor, and the virus band collected by puncturing the bottom of the tube. After the virus band was diluted with an equal volume of 0.01 M Tris pH 8.1, it was layered on a 3-ml linear gradient of density 1.2–1.4 g/ml and centrifuged for 1 hour at 39,000 rpm in a Spinco SW 39 rotor. The principal contaminants remained at, or near, the top of the gradient, while the virus sedimented to its isopycnic level at a buoyant density (ρ) of 1.34. ^{32}P-labeled virus prepared by this method was essentially free of low molecular weight soluble ^{32}P compounds, whereas virus purified by the original method

was still slightly contaminated after three overnight centrifugations. The purified virus was free of identifiable, small, adenoassociated virus (Atchison *et al.*, 1965; Melnick *et al.*, 1965) by electron microscopic examination. Protein was measured by the method of Lowry *et al.* (1951), and the absorbance at 260 mμ was measured on aliquots of virus dissociated with 0.5% SDS in 0.02 M sodium phosphate pH 7.2. It was necessary to use detergent solutions for reliable measurements of ultraviolet absorbance because untreated virus suspensions had a high and variable light scatter. In this way a value of 0.28 mg protein per OD unit at 260 mμ in SDS-buffer was determined. Taking 2.3×10^7 daltons as the molecular weight of virus DNA (Green *et al.*, 1967), which is 13% of virion dry weight (Green and Piña, 1964), and with the above-determined ratio of protein to OD at 260 mμ in SDS, one can calculate that there are 1.1×10^{12} particles per OD_{260} unit.

Highly purified virus for use as inoculum was diluted in 0.1% bovine serum albumin, 0.85% sodium chloride, and 0.01 M Tris-HCl at pH 8.1, and sterilized by filtration through a Millipore type GS or Corning sintered-glass UF-filter at a concentration of 10^{12} physical particles per milliliter. Virus in sterile BSA-Tris-saline was stable for months at 4° (personal communication, Dr. David Axelrod). It was not necessary to dialyze CsCl from the virus before dilution in BSA-saline.

In all cases, except for the preparation of virus stocks (see above), cells to be infected were concentrated to 10^7 per milliliter in Eagle's medium or Puck's saline without serum and inoculated with 200–1000 virus particles per cell. After 15 minutes at room temperature about 30% of the virus was absorbed (Daniels, Maizel, and Scharff, unpublished data using ^{32}P-labeled virus). Assuming 10–20 physical particles per infectious unit (Green and Piña, 1964), 3–6 infectious units were absorbed per cell. The cells were then diluted to 1.5×10^5 cells/ml and incubated with continuous stirring at 37° for 40–48 hours. (Infection of the cells for longer than 20 min. at the initial high density of 10^7/ml caused considerable decrease in the yield of virus.)

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[4] Suggested to us by W. G. Laver, Austrialan National University, Canberra, Australia.

For preparation of radioactive virus, labeled precursors were added to medium lacking the corresponding amino acid. For ^{32}P-labeled virus, medium lacking phosphate was used and the culture was kept in a 5% CO_2–air atmosphere to maintain pH. Normal yields were obtained when 10 mC of orthophosphate-^{32}P was added to 4×10^8 infected cells.

Sodium dodecyl sulfate (SDS) dissociation of virus. Virus suspensions were dialyzed against 0.02 M phosphate buffer pH 7.1 overnight to dilute the CsCl at least 1000-fold. For virus concentrations of 1–2 mg/ml, 10% SDS (prepared from 95% SDS, Matheson, Coleman and Bell) was added to a final concentration of 1–2%; the mixture was made 1% with 2-mercaptoethanol (ME) and allowed to stand overnight at room temperature. At higher concentrations (10–50 mg/ml), dry SDS was added in twice the estimated amount of viral protein. The resulting mixture was dialyzed against 0.01 M sodium phosphate, 0.1% SDS, and 0.1% ME at pH 7 before electrophoresis. Other variations will be described in the context of specific experiments.

Phenol extraction. SDS-dissociated virus at a concentration of 1–10 mg/ml was mixed with an equal volume of water-saturated, redistilled phenol, and shaken for 3–5 minutes by hand in a glass-stoppered tube. After centrifugation at 1200 g for 10 min, the phenol layer (including interphase material and any pellet which may have formed) was withdrawn from below the aqueous layer with a fine Pasteur pipette and ejected into 5–10 volumes of cold acetone-HCl (40 volume acetone: 1 volume N HCl). When this method was used to extract virus labeled in its protein with ^{14}C-amino acids and in its DNA with ^{32}P, all the protein could be recovered in the combined phenol-interphase-precipitate zone, but 70% of the DNA was present as well. Attempts to improve separation of the nucleic acids and proteins by elevated temperature, addition of 8 M urea, 2 M CsCl, or omission of SDS were unsuccessful. Presumably, similar problems led Green and Piña (1964) to treat virus with papain before phenol extraction of DNA. For obvious reasons proteolytic

enzymes were not desirable in these experiments.

Electrophoresis of SDS-dissociated virus. Electrophoresis of SDS-dissociated virus by a discontinuous ("disc") buffer system (Davis, 1964), modified by inclusion of SDS, did not give reproducible patterns. Apparently some of the SDS-protein complexes move faster than, some slower than, and some at the same rate as the buffer boundary. This results in a "stack" of closely spaced components at the buffer discontinuity, the exact configuration of which is sensitive to minor variations in gel concentration, temperature, and possibly other conditions. To eliminate this difficulty a continuous sodium phosphate–SDS buffer system has been employed as previously described (Maizel, 1966). Details of conditions for specific experiments are given in figure captions.

Low pH, 8 M urea electrophoresis of phenol-extracted proteins. Phenol-extracted protein, precipitated with acetone as described above was washed three times with cold acetone-HCl, sedimented at low speeds and taken up in 10 M urea, 10% mercaptoethanol, 0.02 M phosphate pH 7.1 to a concentration of 1–10 mg of protein per milliliter. After 16–24 hours at room temperature, volumes not greater than 0.2 ml were separated on 7.5% acrylamide gels using the system of Reisfeld et al. (1962), modified by inclusion of 8 M urea in the gels. The spacer gel was omitted, since its absence did not affect resolution under these conditions. Gels 5 mm in diameter and 5 cm long were used for staining, and gels 6 mm by 10 or 20 cm were used for fractionation and isotopic analysis. If the gel was stained and not counted for radioactivity, it was immediately placed into 0.3% amido black 10 B (National Aniline Co.) in 7% acetic acid for at least 4 hours, and decolorized either by washing in 7% acetic acid or by the electrophoretic destaining method (Reisfeld et al., 1962; Davis, 1964). When radioactive gels were to be stained and subsequently counted, they were fixed overnight in methanol–acetic acid–water (80:7:13 v/v) prior to staining in order to minimize loss of radioactive protein.

67% acetic acid dissociation and electro-

phoresis. One volume of dialyzed virus in 0.02 *M* phsophate was mixed with 2 volumes of cold glacial acetic acid, and the mixture was subjected to electrophoresis after one-half hour in the cold.

Gels made with 67 % acetic acid were prepared using one volume of Reisfeld's solution "A" (Reisfeld, 1962), two volumes of solution "C" (30 g acrylamide, 0.8 g *N*,*N'*-bismethyleneacrylamide per 100 ml), 6 volumes of glacial acetic acid, 0.006 volume of additional tetramethylethylenediamine, and 0.015 volume of 10 % ammonium persulfate. Sixteen-centimeter tubes were nearly filled with gel solution, stoppered, and allowed to stand overnight at 37°. The surface formed during the slow polymerization of the acetic acid gels was unsuitable because of a poorly defined upper boundary. To obtain a flat surface, a scribe mark was made near the middle of the gel tube, which was then cracked carefully so as to leave the gel unbroken. The exposed gel column was cut with a sharp razor blade to produce a flat surface, and the cut end of the gel and tube was then coupled to a short length of additional glass tubing to provide space for overlaying the sample. Gels were stained in the usual manner with 0.3 % amido black 10 B in 7 % acetic acid.

Dissociation and electrophoresis under alkaline conditions. Virus was dialyzed for several days against 0.1 *M* Na₂CO₃ according to the method of Wilcox and Ginsberg (1963), and the soluble proteins were electrophoretically separated in the standard system of disc electrophoresis (Davis, 1964). Results from this method were variable and are presented only to show that a previously described method also produces a heterogeneous protein pattern.

Radioisotopes and counting procedures. Individual and mixed (RPH) ³H- or ¹⁴C-labeled amino acids were obtained from Schwarz BioResearch Inc. Orthophosphate-³²P was obtained from Oak Ridge National Laboratory. Samples in solution to be counted (e.g., fractions from sucrose gradients or various extraction procedures), were mixed with 0.5 mg of human γ-globulin, and "100 %" TCA (1.0 g TCA per milliliter solution) was added to a final TCA

concentration of 5 %. After 30 minutes in the cold, the samples were filtered onto 25-mm type HA Millipore filters, washed, cemented onto planchets, dried under a heat lamp, and counted on a Nuclear Chicago Model 8700 low background counter (Maizel, 1966). Fractionated gels were counted in a gas flow or scintillation counter as previously described (Summers *et al.*, 1965; Maizel, 1966).

RESULTS

Dissociation and Electrophoresis of Adenovirus Type 2

Adenovirus type 2 was most effectively dissociated into its constituent polypeptide chains by treating with 1–2 % sodium dodecyl sulfate at neutral pH. This results in complete dissociation of viral DNA and protein (Fig. 1). Sucrose gradient centrifuga-
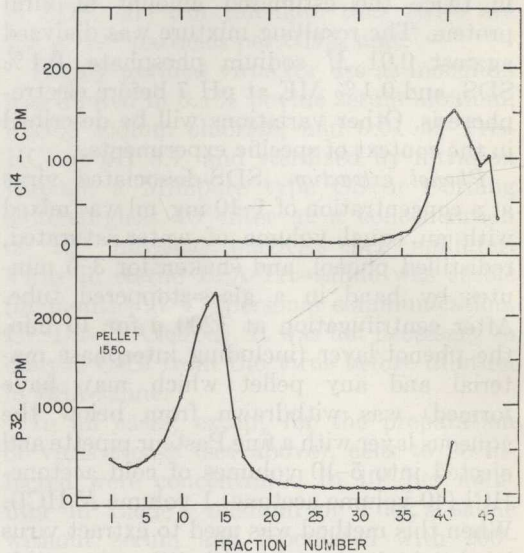

FIG. 1. Dissociation of adenovirus type 2 DNA and protein by SDS. Two separate preparations of purified adenovirus type 2 were prepared, one labeled in its DNA with ³²P (10 mC/4 × 10⁸ infected cells), the other in its protein with lysine-¹⁴C (50 μC/4 × 10⁸ infected cells). Each was dissociated with 2% SDS, layered separately on a 15–30% sucrose gradient made up in 0.5% SDS and 0.02 *M* sodium phosphate buffer (pH 7.1), and centrifuged for 4 hours at 39,000 rpm in the Spinco SW 39 rotor at room temperature. The upper panel shows the sedimentation of ¹⁴C radioactivity while the lower represents that of the ³²P.

tion of SDS-treated virions shows complete separation of the ^{32}P-labeled viral DNA and the ^{14}C-labeled viral protein. Although in this experiment the two virus preparations were centrifuged separately to be certain that there was no small peak of ^{14}C label in the area where the DNA sedimented, similar results were obtained when preparations were either mixed and centrifuged on the same gradient, or when doubly labeled virus preparations were analyzed. This treatment also dissociated the protein into its constituent polypeptide chains, and when followed by electrophoresis on acrylamide gels containing SDS, proved to be the most satisfactory method for the resolution, quantitative measurement, and comparison of viral proteins (Maizel, 1966). Figure 2 shows the electrophoretic pattern of proteins dissociated from purified virus in this manner. A stained gel shown at the top of the figure is to be compared with the pattern of radioactivity of virus proteins labeled with ^{14}C-amino acids (Fig. 2A). The components have been designated II–X for future reference. The presence of certain peaks, which are not prominent in this figure, as well as the presence of multiple peptides in some other peaks, will be validated in subsequent experiments. More than 90 % of the radioactivity applied to the gel could be accounted for in the fractionated samples, and the pattern of the dissociated protein was the same whether the sample was placed immediately on the gel or dialyzed before electrophoresis, indicating that no small components were lost through the dialysis tubing.

All the peaks clearly resolved in Fig. 2A are real and reproducible. They represent discrete polypeptides that form an integral part of the virion. However, certain aspects of the electrophoretic pattern require further comment. (1) If dissociation with SDS is carried out at room temperature, peaks are found on the cathodal (left) side of component II. These peaks, shown in Fig. 2B, are present in variable amounts in different experiments, are sometimes eliminated by prolonged dialysis against 0.1 % SDS at room temperature and by treatment with SDS at 37° for 30 min, and are more prom-
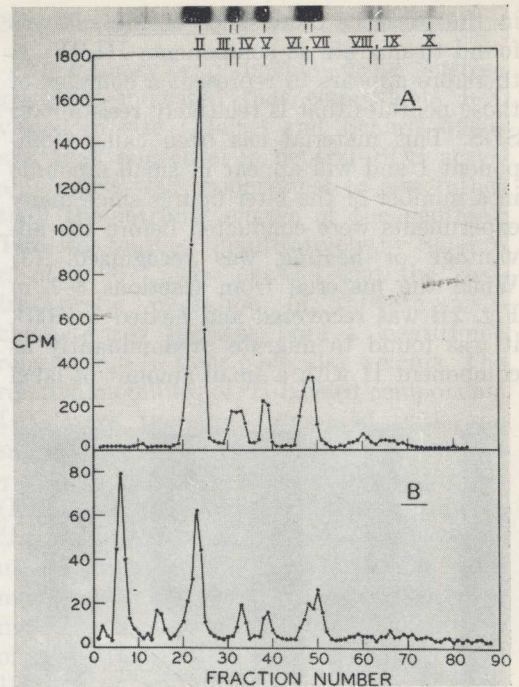

FIG. 2. Electrophoresis of SDS-dissociated adenovirus type 2 in neutral SDS-acrylamide gels. Purified adenovirus type 2 labeled with mixed ^{14}C-amino acids was dissociated with SDS and mercaptoethanol for 30 min at room temperature (B) or for 1 min at 100° (A and stained gel). The stained gel (upper panel) was 5 mm in diameter by 10 cm long, and was run for 2 hours at 6 mA per gel (i.e., 3 V/cm). The gels fractionated and counted for radioactivity (A + B) were 6 mm in diameter by 20 cm long, and were run simultaneously for 16 hours at 6 mA per gel (i.e., 2.5 V/cm). The radioactive peaks in panel A have been aligned with a photograph of the stained gel. Since regions VIII–IX and X of the stained gel were easily seen by eye, but photographed poorly, they were retouched on the photograph (in this figure only). Migration is toward the anode on the right.

inent if the purified virus had been stored for long periods of time. These slowly migrating peaks can, however, be completely eliminated by heating the virus preparation at 100° for 1 min in the presence of 1–2 % SDS and mercaptoethanol (Fig. 2A), under which conditions no qualitatively new peaks appear. (2) When the material migrating in fractions 13–17 in Fig. 2B was recovered from gels in SDS and heated at 100° either

in the presence or absence of ME, it was found to migrate as components III–IV. It therefore appears to represent a complex of those peptides that is relatively resistant to SDS. This material has been called component I and will appear in small amounts in a number of the later figures since many experiments were conducted before the advantage of heating was recognized. (3) When the material from fractions 3–7 in Fig. 2B was recovered and heated to 100°, it was found to migrate predominantly as component II with a small amount of label
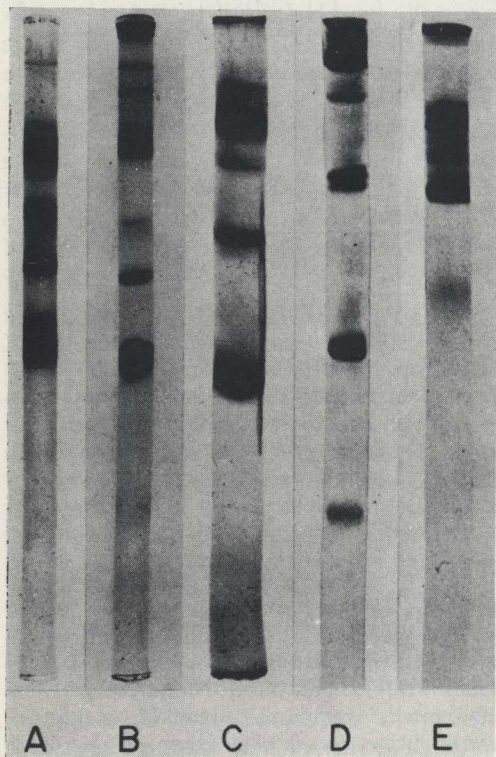
FIG. 3. Electrophoresis of adenovirus type 2 dissociated by a variety of methods. (A) Dissociation with hot SDS and mercaptoethanol, and electrophoresis in neutral SDS. (B) Dissociation with SDS and mercaptoethanol at room temperature and electrophoresis in neutral SDS. (C) Dissociation and electrophoresis in 67% acetic acid. (D) Dissociation with phenol-SDS and electrophoresis in 8 M urea at pH 4.3. (E) Dissociation and electrophoresis under alkaline conditions. Details of sample preparation are given in Methods. Migration was from top to bottom. The anode is at the bottom in A, B, and E, and at the top in C and D.

also found in regions VIII and IX. In unpublished experiments with a variety of proteins it has become clear that treatment with SDS at 100° for 1 min does not cleave peptide bonds. For example, the electrophoretic pattern of γ-globulin, its polypeptide chains, and DEAE-purified hexons (Maizel *et al.*, 1968) is unchanged when the samples are treated with SDS at 100°. Although the exact nature of the bonds joining these component polypeptides is not clear at this time, the relative absence of this material in some preparations and its complete conversion to other known peptides of smaller molecular weight upon heating has led us to conclude that they represent aggregates, and not single polypeptide chains. Preparations heated and then maintained at ambient temperatures do not reaggregate. The possible significance of these aggregates in terms of virus structure is discussed in the following paper (Maizel *et al.*, 1968). (4) A minor, faster-moving component on the anodal side of II is also variable, and where it has been seen, the virus had usually been stored at high concentration. (5) In stained gels containing large amounts of protein, faint bands can often be seen in the regions between the major peaks. In addition, there is a very sharp stained band at fraction 11 of Fig. 2A that shows clearly on the stained gel, but contains insignificant amounts of radioactivity. All these components are quantitatively so minor that it is not possible to consider them in the present discussion, or to ascribe a role to them in virus architecture.

Against the possibility that the polypeptide heterogeneity of type 2 adenovirus was an artifact peculiar to the neutral-SDS system, virus was dissociated by several widely different procedures and examined in different systems of electrophoresis. In Fig. 3, gel A is a neutral-SDS gel of heated, SDS-dissociated virus similar to that shown in Fig. 2. Gel B is unheated, SDS-dissociated virus on a neutral-SDS gel. The zones due to aggregated peptides are clearly seen near the top of this gel. Gel C is 67% acetic acid-dissociated virus migrated on a gel containing 67% acetic acid. Gel D is phenol-SDS treated virus proteins after migration
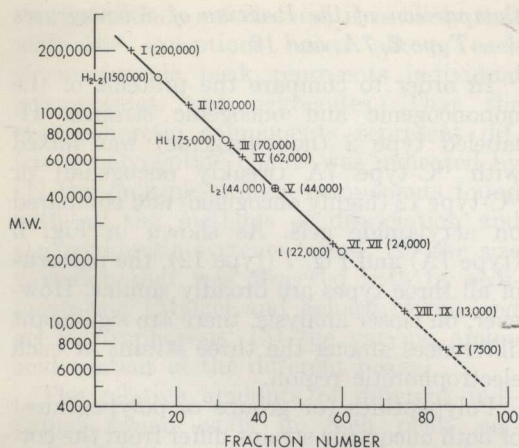
FIG. 4. Estimation of molecular weight of adenovirus type 2 proteins by neutral-SDS gel electrophoresis. ³H-labeled virion proteins and ¹⁴C-labeled immunoglobulins were mixed, dissociated, and fractionated in a 0.6 × 20 cm neutral SDS, 5% acrylamide gel. The position of each ³H- and ¹⁴C-labeled peak was determined by scintillation counting. The ¹⁴C-labeled immunoglobulins were taken as the standards against which the molecular weight of each ³H-labeled viral polypeptide was determined (Shapiro et al., 1967).

in an 8 M urea, pH 4.3 gel. Gel E shows the pattern obtained from virus dissociated at pH 10.5 without detergent (Wilcox and Ginsberg, 1963) and analyzed with the standard pH 8.3 system of "disc" electrophoresis. It is clear that there is considerable heterogeneity in all these systems, whether dissociation is conducted with or without detergents, and irrespective of whether migration is anodal or cathodal. Procedures using neutral SDS have been used most extensively in the present experiments because they provide good solvent action with mild chemical treatment in which it is unlikely that covalent bonds, other than disulfide bonds, are affected; in the case of disulfide bonds, breakage or maintenance can be controlled by the presence or absence of mercaptoethanol. Separation is mainly dependent on size rather than charge (Shapiro et al., 1967). Resolution is such that the system can handle larger quantities of more complex mixtures than any other electrophoresis system so far tested with fewer attendant problems and artifacts than with most other systems.

Size Heterogeneity of Adenovirus Peptides

Preliminary experiments using sucrose gradients indicated that components I–X varied in size (Maizel and Scharff, 1966) and that migration in neutral- SDS acrylamide gels was proportional to size rather than the intrinsic charge of the peptides. This was verified quantitatively by Shapiro et al. (1967), who showed that the electrophoretic migration of thirteen well-characterized proteins bore a logarithmic relationship to their molecular weights. The relative mobilities of ³H-labeled components I–X were therefore compared with the same preparations of ¹⁴C-labeled mouse 7 S γ-globulin (H₂L₂), γ-globulin half molecules (HL), disulfide-linked light-chain dimers (L₂), and single light chains (L) as had been used by Shapiro et al. (1967) (Fig. 4). The migration of each of these gamma globulin molecules is linearly related to the logarithm of their molecular weights (Fleischman, 1966); and the molecular weights for the adenovirus proteins have been interpolated from that reference curve (Fig. 4). They vary in size from a MW 120,000 for II, through 28,000–30,000 for VI and VII, to approximately 13,000 for the minor but reproducible components VIII and IX. This range of polypeptide size is not unusual. when the linear plot is extrapolated to the migration rate of I and slower components, the molecular weights are extremely large, again suggesting that they are composed of aggregates of lower molecular weight polypeptides (see above).

Evidence that the Electrophoretic Components Differ in Composition

On examining the ratios of radioisotope when two different amino acids (lysine-³H and valine-¹⁴C on the bottom panel of Fig. 5) were incorporated, the widely varying compositions of the polypeptides is evident. The data also confirm the presence of multiple proteins in regions III–IV and VI–VII. The variation in the ratio of the two amino acids across these peaks is to be contrasted with the control (Fig. 5, upper panels) in which the ³H/¹⁴C-lysine ratio is constant throughout the peak. Table 1 summarizes the distribution of label in
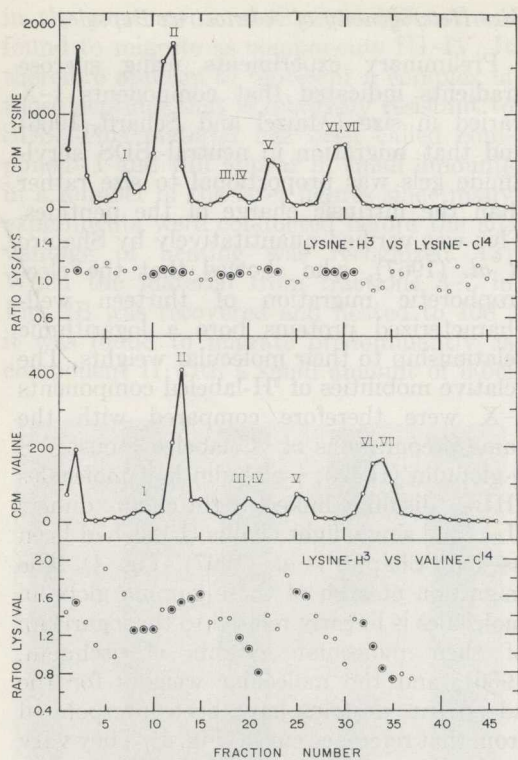
Fig. 5. Differences in the relative amounts of lysine and valine among the different proteins of adenovirus type 2. Virus isolated from cells infected in the presence of either a mixture of lysine-^{3}H and lysine-^{14}C (upper panels), or lysine-^{3}H and valine-^{14}C (lower panels) was dissociated, electrophoretically separated, and the ratio of ^{3}H to ^{14}C determined in each fraction of the gel by liquid scintillation counting. The anodal direction is to the right. Gels were 0.5 × 10 cm, 5% acrylamide, and neutral SDS. The topmost pattern is lysine-^{14}C radioactivity from the combined ^{3}H- and ^{14}C-labeled lysine control. The third panel from the top is the valine-^{14}C radioactivity from the combined lysine-^{3}H and valine-^{14}C gel. The ratio of peak fractions are shown as encircled points.

electrophoretic components when preparations of virus labeled with several different amino acids were analyzed. There are considerable variations in the distributions depending on the amino acid chosen, as would be expected for polypeptides of different composition, and all polypeptides possess significant amounts of all the amino acids tested.

Comparison of the Proteins of Adenoviruses Type 2, 7A, and 12

In order to compare the proteins of the nononcogenic and oncogenic strains, ^{3}H-labeled type 2 (nononcogenic) was mixed with ^{14}C-type 7A (weakly oncogenic) or ^{14}C-type 12 (highly oncogenic) and compared on acrylamide gels. As shown in Fig. 6 (type 7A) and Fig. 7 (type 12), the patterns of all three types are broadly similar. However, on closer analysis, there are significant differences among the three strains at each electrophoretic region.

Polypeptides (or groups of polypeptides) of both oncogenic strains differ from the corresponding polypeptides of type 2, and differ from each other. Differences in size or composition or both may contribute to these differences in mobility, through affecting the frictional properties, the intrinsic charge, or the resultant charge of the SDS-protein complexes. However, it is pertinent to note that differences in peptides significant enough to cause detectable differences in electrophoretic migration must reflect quite major intertypic differences in the corresponding genes.

DISCUSSION

It has been shown by a variety of dissociation and fractionation procedures involving SDS, 10 M urea, 67% acetic acid, or pH 10.5 that the protein of highly purified adenovirus type 2 consists of a number of component polypeptides. Of all the procedures tried, treatment with SDS at neutral pH has proved to be the most satisfactory since it completely releases the DNA from the protein and at the same time dissociates the protein into a number of peptide chains separable on SDS-containing acrylamide gels. This technique, which discriminates primarily according to size (Shapiro *et al.*, 1967), has revealed that the adenovirion contains a minimum of nine different components.[5] Since the dissociation was carried out under conditions in which

[5] Any such fractionation procedure can only be regarded as giving a minimum estimate of the number of components until homogeneity of each component is demonstrated by other means such as partial sequence analysis.

disulfide bonds are cleaved, it is likely that with the exceptions noted above, each electrophoretic peak represents individual polypeptides, not aggregates. That the electrophoretic components represent different polypeptide chains was indicated by (1) the multiple protein components found with all the methods of dissociation and electrophoretic systems tested; (2) the size variation that was observed both by sedimentation (Maizel and Scharff, 1966) and gel electrophoresis; (3) the varying amino acid content of the different peaks.

The relative amounts of different individual amino acids in each peak suggests that some of these peaks, such as VI–VII, in fact consist of at least two different polypeptide chains. None of the components lacked any of the individual amino acids investigated (lysine, valine, threonine, arginine, and tryptophan). Peptides V, VI, VII were somewhat enriched for arginine, but unlike histones, contained tryptophan as well (Bonner and T'so, 1964).

Electrophoretic comparison of the polypeptides of adenovirus type 2 (nononcogenic), 7A (weakly oncogenic), and 12 (highly oncogenic) revealed a general similarity of pattern in all three types, but at the same time distinct and significant differences between nearly all the corresponding components. (This incidently supports the argument that the nine different components of virions represent distinct polypeptides.)

TABLE 1

DISTRIBUTION OF RADIOACTIVITY IN POLYPEPTIDES FROM VIRUS LABELED WITH DIFFERENT
AMINO ACIDS

| Type of radioactive precursor | Ratio[a] of radioactivity in various regions to that in region II and percentage[b] distribution of radioactivity in various electrophoretic components | | | | | | % recovered[e] as II-X |
	II[c]	III, IV[d]	V	VI, VII	VII, IX	X	
^{14}C-mixed amino acids (RPH)	1.0^a $(53.5)^b$	0.16^a $(8.5)^b$	0.10^a $(5.2)^b$	0.26^a $(13.5)^b$	0.065^a $(3.5)^b$	—	84.2
(RPH)	1.0 (48.9)	0.17 (8.5)	0.11 (5.2)	0.28 (14.1)	0.067 (3.3)	0.0082^a $(4.0)^b$	83.8
(RPH)	1.0 (52.2)	0.17 (8.9)	0.11 (5.7)	0.28 (14.1)	—	—	—
Lysine-^{3}H	1.0 (53.5)	0.16 (8.3)	0.11 (5.9)	0.46 (24.8)	0.035 (1.9)	0.022 (1.2)	95.6
Tryptophan-^{14}C	1.0 (57.5)	0.16 (8.9)	0.052 (3.0)	0.37 (21.4)	0.064 (3.7)	0.042 (2.4)	96.9
Arginine-^{14}C	1.0 (33.2)	0.27 (8.9)	0.24 (7.9)	1.08 (35.8)	0.13 (4.5)	0.20 (6.8)	97.1
^{14}C-labeled threonine and valine	1.0 (50.3)	0.24 (11.9)	0.14 (7.1)	0.32 (16.3)	0.11 (5.6)	0.05 (2.5)	91.7

[a] The radioactivity in a given region was divided by that of region II from the same gel.

[b] Number in parentheses, expressed as percent of total counts recovered from the gel. Total recovery was 90% or better.

[c] In the gel samples dissociated with SDS at room temperature, radioactivity near the origin (cf. Fig. 1B) was included as region II.

[d] In gels as in (c) above the radioactivity in region I (cf. Figs. 1B, 4, and 6) was added to region III–IV.

[e] Sum of radioactivity in peak regions II through X expressed as a percentage of the total radioactivity recovered in the gel. With mixed amino acid (RPH)-labeled proteins, a higher fraction of the radioactivity was recovered in the major peaks than when single amino acids were used.
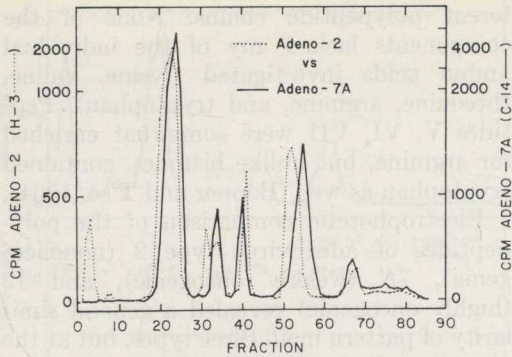
FIG. 6. Comparison of the proteins of adenovirus types 2 and 7A. Adenovirus type 2 labeled with threonine-^{3}H and valine-^{3}H was mixed with adenovirus type 7A labeled with threonine-^{14}C and valine-^{14}C, dissociated with SDS and mercaptoethanol, and electrophoretically separated as in Fig. 1.
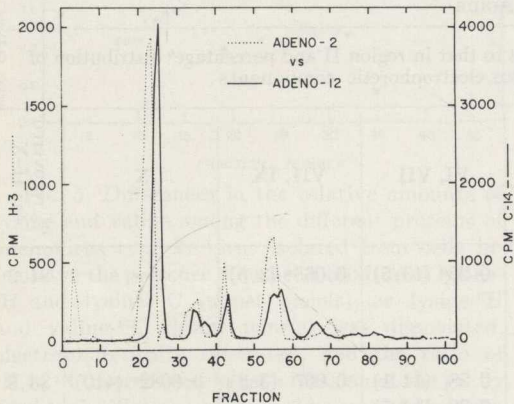

FIG. 7. Comparison of the proteins of adenovirus types 2 and 12. Adenovirus type 2 labeled with threonine-^{3}H and valine-^{3}H was mixed with adenovirus type 12 labeled with threonine-^{14}C and valine-^{14}C, dissociated with SDS, and mercaptoethanol, and electrophoretically separated as in Fig. 1.

The appearance of a detectable doublet of proteins from type 12 in the VI–VII region (Fig. 7) confirms the heterogeneity detected by isotope ratio analysis of type 2 virions (cf. Fig. 5). There is no obvious present correlation between the peptide pattern and the oncogenicity of the several types tested. However, it is pertinent to note that there are apparently differences in most, if not all, of the virion protein genes of types 2, 7A and 12.

A detailed discussion of the relationship of the individual peptides to the architecture of the virion will be presented in the following paper.

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The Polypeptides of Adenovirus

II. Soluble Proteins, Cores, Top Components and the Structure of the Virion[1]

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The adenovirion has been shown to have a much more complex structure than was previously assumed. Eight different types of polypeptides have been assigned a place in the particle. Three are present in an outer capsid, three in an inner core, and two others in association with hexons.

The hexon capsomere is composed of about three molecules of a single type of peptide of molecular weight (MW) 120,000 which comprises about 50% of the total virion protein. Groups of such capsomeres, released from the virion by 5 *M* urea, are found to be associated with two minor polypeptides, each of MW 13,000.

The penton-base is composed of a single type of peptide of MW 70,000, and the fiber is composed of another peptide of MW 62,000.

The internal core released by treatment of the virion with 5 *M* urea, contains the viral DNA in association with three arginine-rich peptides of MW 44,000, 24,000, and 24,000, comprising some 20% of the total virion protein. The same three peptides are relatively lacking in the "empty" capsids found as the "top components" of cesium chloride gradients of crude virus preparations.

INTRODUCTION

Electron microscopic studies by a number of investigators (Horne *et al.*, 1959; Wilcox *et al.*, 1963; Valentine and Pereira, 1965; Smith *et al.*, 1965; Norrby, 1966a,b), have shown the adenovirion to have an icosahedral form the surface of which is constructed of 252 capsomeres. Two hundred and forty of the capsomeres are of a type called "hexons" (Ginsberg *et al.*, 1966), each of which is surrounded by six neighboring capsomeres. At each of the twelve vertices of the icosahedron there are complex cap-

somores that have been called "pentons." They consist of a basal unit ("penton base") from which a "fiber" about 200 Å long projects radially. The "fiber" appears to have a globular termination (Valentine and Pereira, 1965). These surface substructures also exist in the infected cell as the unassembled "hexon," "penton" (penton base and fiber), and "fiber" soluble viral antigens (Klemperer and Pereira, 1959; Philipson, 1960; Wilcox and Ginsberg, 1961; Valentine and Pereira, 1965; Ginsberg *et al.*, 1966).

In the preceding paper (Maizel *et al.*, 1968), methods were described for the degradation of adenovirions into their constituent polypeptide chains and for the separation and characterization of these polypeptides on acrylamide gels. At least nine unique peptides were recognized. In this paper, the location in the virion of most of the nine peptides will be established by comparing their electrophoretic migration
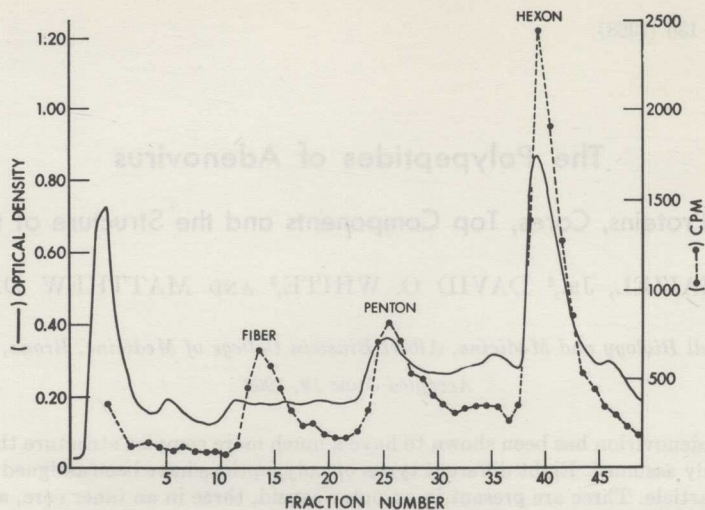
Fig. 1. Separation of fibers, pentons, and hexons by chromatography on DEAE-cellulose. Adenovirus type 2-infected HeLa cells, labeled with valine-^{14}C and threonine-^{14}C between 20 and 30 hours postinfection, were washed, lysed, and loaded onto a 1.2 × 12-cm DEAE-cellulose column. Elution was conducted with a continuous gradient from a nine-chambered gradient mixer. Successive chambers each contained 40 ml of sodium chloride at concentrations of 0, 0, 0.1, 0.2, 0.3, 0.35, 0.6, 0.8, 0.8 M in 0.01 M sodium phosphate buffer (pH 7.1).

with that of the peptides found in: (1) the known soluble viral antigens, (2) mildly degraded virions, (3) "top components" from CsCl gradients of infected-cell extracts.

MATERIALS AND METHODS

Most of the Materials and Methods used in this work have been described in detail in the preceding paper (Maizel *et al.*, 1968).

DEAE-cellulose chromatography. Extracts of radioactively labeled, infected cells were prepared for DEAE-cellulose chromatography by extraction of 5 to 10 × 10^7 cells in 10–20 ml of 0.01 M Tris buffer twice with an equal volume of Freon 113 (duPont). After dialysis against starting buffer (0.01 M sodium phosphate pH 7.1), samples were chromatographed on 1.2 × 12 cm columns of washed DEAE-cellulose (Schleicher and Schuell). A continuous linear gradient was formed with a 9-chamber variable gradient mixer (Phoenix Instrument Company) containing the buffers shown in the caption of Fig. 1. The buffer was pumped through the column at a rate of 36 ml/hour with a Technicon proportion-

ing pump. The eluate was monitored for absorbancy at 280 mμ and for trichloroacetic acid-precipitable radioactivity as described previously (Maizel *et al.*, 1968).

Preparation of virus "cores." Preparations containing 1–5 mg of purified virus per milliliter were dialyzed against 0.01 M sodium phosphate buffer (pH 7.1) to remove CsCl. Urea was added to a final concentration of 7.2 molal (5 molar). Within 5–10 minutes the opalescence due to light scattering by virions decreased, but did not completely disappear. The urea-treated virus was then redialyzed against 0.01 M phosphate buffer (pH 7.1) and analyzed by centrifugation through a 5–20% (w/w) sucrose gradient containing the same buffer at 24,000 rpm in the Spinco SW 25.3 rotor for 3 hours.

RESULTS

Electrophoretic Characterization of Soluble Viral Antigens

Adenovirus type 2-infected HeLa cells were labeled with valine and threonine-^{14}C late in the multiplication cycle (from 20 to 30 hours postinfection), when relatively

little cell protein was being synthesized (Ginsberg *et al.*, 1967; White *et al.*, 1968). The cells were washed, lysed, and subjected to DEAE-cellulose chromatography. The elution pattern is shown in Fig. 1.

Three major peaks of radioactivity are clearly recognizable. They correspond closely with those previously obtained by Klemperer and Pereira (1959), Philipson (1960), Wilcox and Ginsberg (1961), Berman and Rowe (1965), and Gelderblom *et al.* (1965). These three components have been well characterized morphologically and immunologically by Wilcox *et al.* (1963) and Valentine and Pereira (1965). Fractions 12–16 correspond to the "fiber"; fractions 24–26 to the "penton" capsomere ("penton base" plus "fiber"); fractions 39–44 to the "hexon" capsomere.

The electrophoretic characteristics of the ^{14}C-labeled proteins of the viral subunits found in each of the major DEAE peaks was compared with that of virions by mixing material obtained from a single cycle of DEAE chromatography with ^{3}H-labeled virions, dissociating with SDS, and analyzing the material on SDS-containing acrylamide gels. Figure 2 shows the electrophorogram of the hexon capsomere,
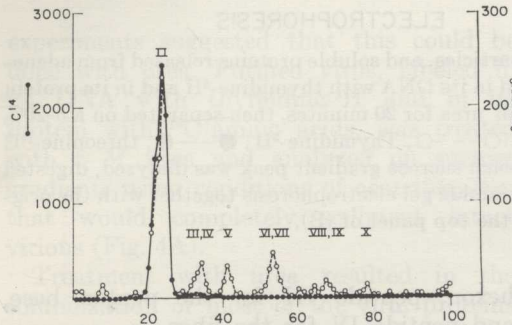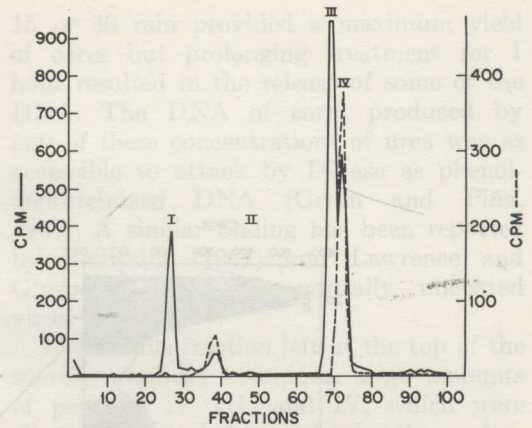

FIG. 3. Polypeptide composition of fibers and pentons of adenovirus type 2. Fibers (fraction 14) and pentons (fraction 25) were obtained from chromatograms (cf. Fig. 1) of extracts labeled with threonine-^{14}C and valine-^{14}C, mixed with ^{3}H-amino acid labeled virus and analyzed by gel electrophoresis as in Fig. 2 except that dissociation of the sample with neutral SDS and mercaptoethanol was done at room temperature and electrophoresis was for 16 hours at 10 mA per gel. The resultant patterns of polypeptides were plotted together for comparison. The peak positions of known virion components are designated by the appropriate Roman numerals (see Fig. 1). Recall that components I and the unmarked component between I and II are converted to III and IV by SDS at 100° for 1 minute and therefore are not distinct peptides. – – –, Fiber; ——, penton.

FIG. 2. Polypeptide composition of the hexons of adenovirus type 2. ^{14}C-labeled hexon capsomeres, eluted from a DEAE-cellulose column (Fig. 1, fraction 40) were mixed with ^{3}H-labeled virions. The mixture was dialyzed against 0.01 M sodium phosphate buffer (pH 7.1) made 1% with SDS and mercaptoethanol, heated at 100°C for 1 minute, and analyzed by acrylamide gel electrophoresis as previously described (Maizel *et al.*, 1968). Electrophoresis was for 16 hours at 6 mA on a gel 0.6 × 20 cm containing 5% acrylamide, 0.1% SDS, and 0.1 M sodium phosphate.

which is seen to consist entirely of viral peptide II.

The electrophoretic pattern of the "penton" is shown as a solid line in Fig. 3. It contains two major peptides, which have been resolved by running the electrophoresis at higher voltage and correspond to the viral components III and IV. As this experiment was performed before it became routine practice to heat all samples in the presence of SDS, there are also two slowly migrating minor peaks, now known to be artifacts, which disappear on heating to 100° for 1 minute. These particular peaks were also shown to disappear after heating of SDS-treated whole virus (Maizel *et al.*, 1968).

Fibers prepared by DEAE-cellulose chromatography contained only one major component, which migrated with viral
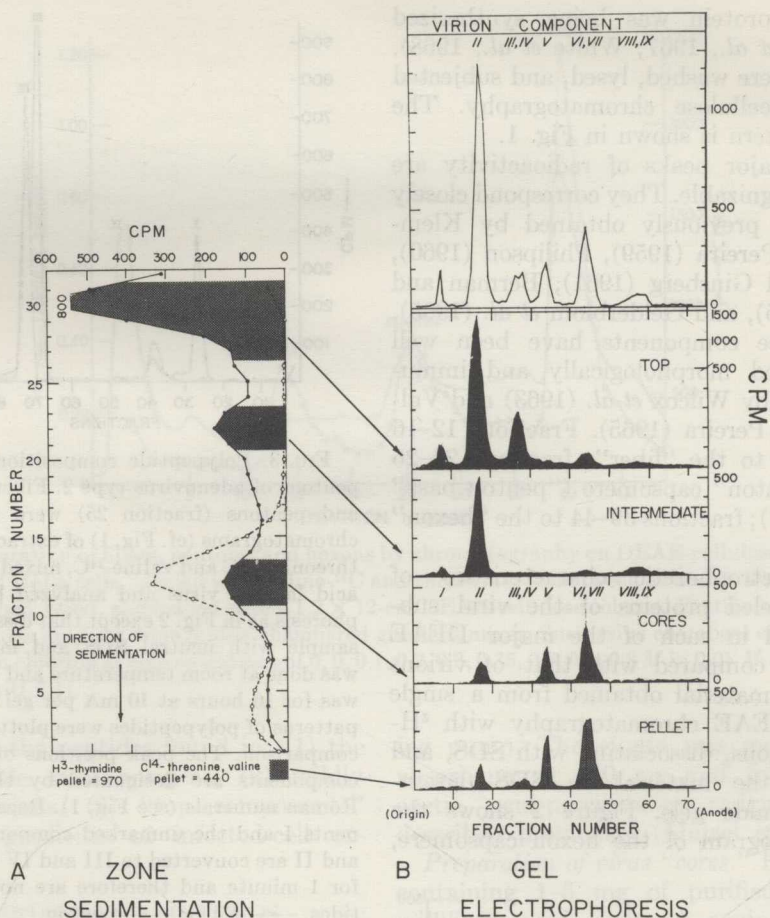
FIG. 4. Polypeptide composition of cores, smaller particles, and soluble proteins released from adenovirus type 2 virions by 5 M urea. Purified virus, labeled in its DNA with thymidine-³H and in its protein with threonine-¹⁴C and valine-¹⁴C, was treated with 5 M urea for 20 minutes, then separated on a 5–20% (w/w) sucrose gradient at 24,000 rpm for 3 hours (A). O---O, Thymidine-³H; ●——●, threonine-¹⁴C and valine-¹⁴C. Material from the central fraction of each sucrose gradient peak was dialyzed, digested with SDS and mercaptoethanol and analyzed by acrylamide gel electrophoresis together with the original virus (B). Proteins of whole virus are shown in the top panel of (B).

peptide IV (Fig. 3 dotted line). Here again, a minor component was present that could be eliminated by heating. Neither penton nor fiber contained any trace of the major constituent of purified virions (peptide II), nor of peptides V–X (when subjected to gel electrophoresis for the standard time).

These experiments suggested that each of the major viral antigens, corresponding to the substructures seen on the surface of the virus, is made up of a single major type of peptide chain: peptide II for the hexon, peptide III for the penton base, and peptide IV for the fiber.

Preparation and Analysis of Virus Cores

Since the three peptides making up the surface proteins of the virion accounted for only 60 % of the total viral protein, it seemed likely that the remaining peptides might be internal problems. Accordingly, gentle methods of virus degradation were explored with a view to partially degrading the virion and revealing an internal core. Previous
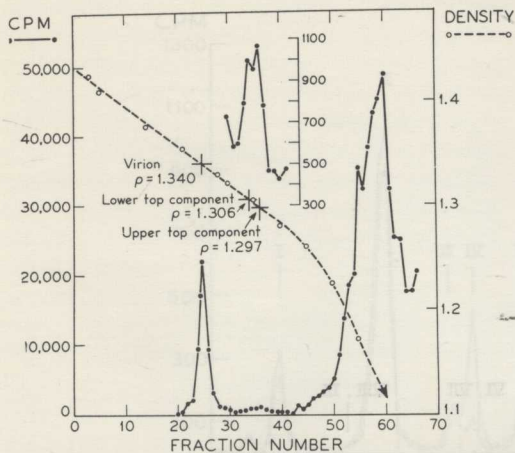
FIG. 5. Separation of "top components" from adenovirus type 2 by cesium chloride gradient centrifugation. A Freon-treated extract of infected cells labeled with ^{14}C-amino acids was layered on a 20 ml preformed CsCl gradient ranging in density from 1.2 to 1.4 g/ml in 0.01 M Tris-HCl pH 8.1, and centrifuged for 3 hours at 25,000 rpm in a SW 25.1 Spinco rotor. Fractions were collected from a puncture in the bottom of the tube and analyzed for trichloroacetic acid-precipitable radioactivity (Maizel et al., 1968) ●——●, and density (refractive index), ○- - -○. The inset shows a 30-fold enlargement of the graph of radioactivity from that region of the gradient containing the top components.

experiments suggested that this could be done with urea. Purified virus, labeled in its DNA with thymidine-^{3}H and in its protein with ^{14}C-amino acids, was treated with 5 M urea and analyzed on sucrose gradients using conditions of centrifugation that would completely sediment intact virions (Fig. 4A).

Treatment with urea resulted in the solubilization of most of the viral proteins (Fig. 4A, fractions 26–32) and the release of two types of sedimentable particles, one of which contained only protein (fractions 21–24), while the other contained both DNA and protein (fractions 10–15) and will be designated "cores." While the yield of cores was approximately the same at any concentration of urea between 5 and 9 molal, treatment with 7.2 molal (5 M) urea at room temperature gave the best yield of the particles containing only protein. Treatment with 7.2 molal urea for

15 or 30 min provided a maximum yield of cores but prolonging treatment for 1 hour resulted in the release of some of the DNA. The DNA of cores produced by any of these concentrations of urea was as accessible to attack by DNase as phenol-deproteinized DNA (Green and Piña, 1964). A similar finding has been reported by Philipson (1967) and Lawrence and Ginsberg (1967) for partially uncoated virus.

The soluble fraction left at the top of the sucrose gradient contained large amounts of peptides II, III, and IV, which were shown in Figs. 1–3 to comprise the surface subunits of the viral capsid.

The slowly sedimenting particles lacking DNA contain mostly peptide II but also have the same amounts of peptide VIII and IX, relative to peptide II, as are found in whole virions. It is of interest that aggregates of these same peptides (II, VIII, IX), resistant to disruption by SDS at room temperature were observed previously (Maizel et al., 1968).

The predominant peptides of the DNA-containing cores are V, VI, and VII. These peptides either are absent or are present in relatively low amounts in both the particles lacking DNA (fractions 21–24) and the soluble proteins (fractions 26–32). The small amounts of peptide II associated with the cores may be significant but equally may reflect contamination, since peptide II makes up half the protein of the original virion.

The pelleted material contained the same peptides as the cores in approximately the same proportions, suggesting that it indeed consists of aggregated cores. This interpretation is supported by the finding that the pellet could be eliminated and the yield of cores increased by incorporating 5 M urea in the sucrose gradient.

Structure of the Top Components of Adenoviruses Types 2 and 7A

When threonine-^{14}C- and valine-^{14}C-labeled adenovirus type 2 was purified from Freon extracts of infected cells on CsCl gradients, the virus band (which could be visualized by illumination with
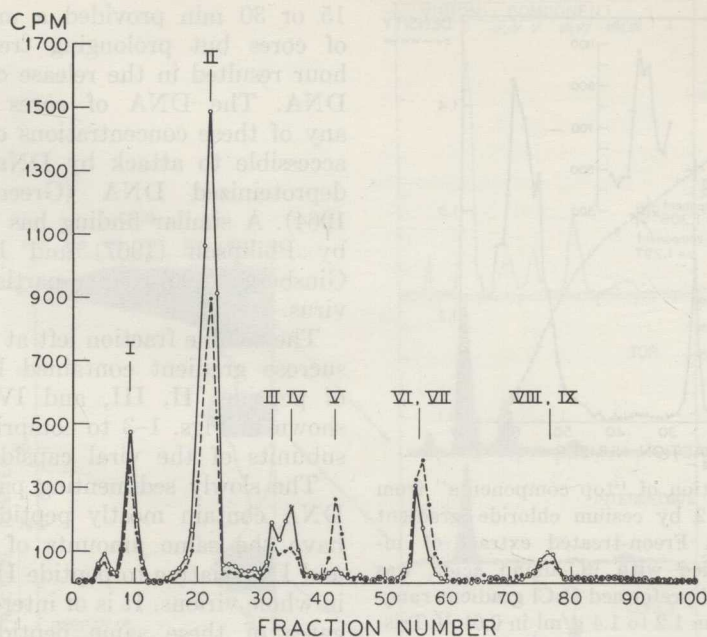
FIG. 6. Polypeptide composition of the adenovirus type 2 "top component" ($\rho = 1.306$). The "top component" of $\rho = 1.306$ was collected from a gradient similar to that shown in Fig. 5, then diluted 1:2 and recentrifuged on a CsCl gradient of $\rho = 1.20$–1.35 for 1 hour at 38,000 rpm in an SW 39 Spinco rotor. Fractions were collected from the bottom of the tube, dialyzed, mixed with threonine-^{3}H- and valine-^{3}H-labeled virus, dissociated, and analyzed electrophoretically as in Fig. 4B. O——O, ^{14}C-top component $\rho = 1.306$; ●---●, ^{3}H-adenovirus type 2.

oblique light in a dark room) was found at a density of 1.340 (Fig. 5). Just above the virus band two other faint but distinct bands could also be seen. These so-called "top components," with measured densities of 1.306 and 1.297, respectively (Fig. 5), were also demonstrable by TCA precipitation of radioactive protein (Fig. 5, inset). When virus labeled only with radioactive thymidine was examined, no comparable peaks could be detected; traces of radioactive thymidine in that region were found on recentrifugation entirely within the major virion band. It was concluded that these "top components" contain no viral DNA. Although we have not examined the top components by electron microscopy, by analogy with another icosahedral virus (Maizel et al., 1967) their peptide structure, density, and lack of nucleic acid suggests that they are empty capsids, as described by Köhler (1962).

After rebanding, the peptide composition of each of the ^{14}C-amino acid-labeled top components was compared with that of

^{3}H-labeled virions on acrylamide gels (Figs. 6 and 7). Both top components show a relative lack of peptide V, and perhaps of peptide VII. Indeed a number of other qualitative and quantitative differences between virions and the top components are evident in the V through VII area of the gel. It is these peptides that are the major constituents of the nucleoprotein core (Fig. 5).

The pooled top components of type 7A were also compared with type 2 virions, and peptide V was again found to be greatly decreased (Fig. 8). Comparison of type 7A top components with type 7A virions can be made by aligning the ^{3}H-labeled type 2 peptides of Fig. 8 with their opposite numbers in Fig. 6 of the preceding paper (Maizel et al., 1968). It will be seen that the major differences between type 7A virion and its top components are analogous to those demonstrated for type 2. Comparison of type 12 virions and top components, although not shown here, gave similar results.
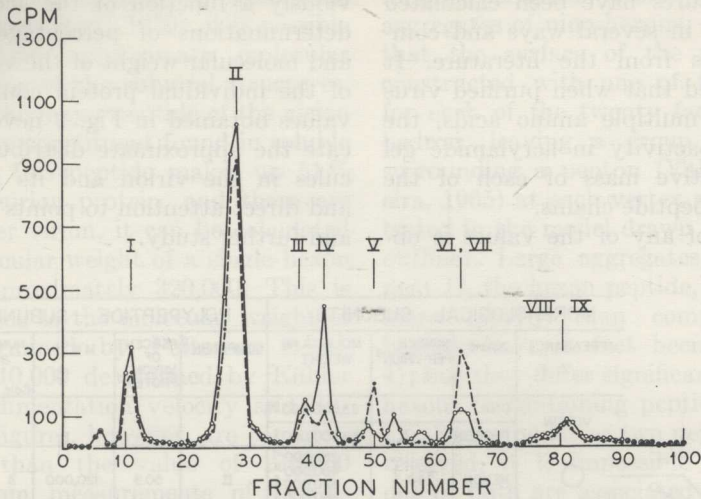
FIG. 7. Polypeptide composition of the adenovirus type 2 "top component" ($\rho = 1.297$). The top component of $\rho = 1.297$ was isolated, rebanded, and analyzed as in Fig. 6. $\bigcirc$——$\bigcirc$, ^{14}C-top component $\rho = 1.297$; $\bullet$---$\bullet$, ^{3}H-adenovirus type 2.
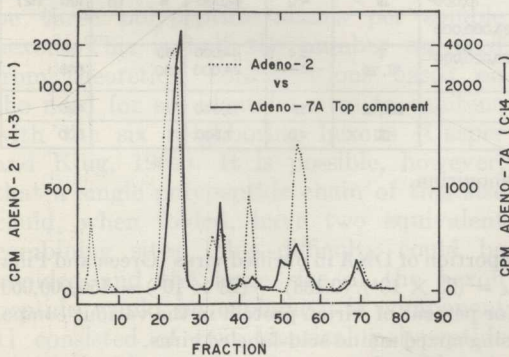

FIG. 8. Polypeptide composition of the adenovirus type 7A "top components." The combined top components from a preparation of threonine-^{14}C and valine-^{14}C-labeled, adenovirus type 7A-infected cells were isolated from a single centrifugation as described in Fig. 5 and were analyzed by electrophoresis as in Fig. 6 and 7. ——, adenovirus-^{14}C type 7A top components; $\bullet$···$\bullet$, adenovirus-^{3}H type 2.

DISCUSSION

In this and the preceding paper the adenovirus type 2 virion has been shown to contain at least nine different polypeptide chains. By the electrophoretic analysis of (a) intact virions, (b) soluble viral antigens, (c) products of mild degradation of whole virus, and (d) naturally occurring top components, combined with the morphological studies of others (Valentine and Pereira, 1965), it has been possible to establish tentatively the location of most of the polypeptide chains found in the virion.
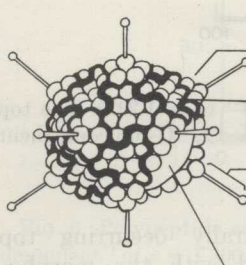
Peptides II, III, and IV are the major components of the hexon, penton base, and fiber, respectively, and they make up most of the outer coat of the virion. Peptides V, VI, and VII appear to be more closely associated with the viral DNA and are the major constituents of the inner core. While only nine distinct peptides have been resolved on the acrylamide gels, this is a minimal number, since certain of the electrophoretic components could conceivably be made up of more than one peptide. This possibility can be resolved only by more rigorous analysis of each component.

A summary of the molecular weights of individual electrophoretic components and their percentage contribution to the virion protein, along with some pertinent data of others, is presented in Fig. 9. The physical and chemical characteristics of the structural antigens of all adenovirus types are completely summarized in a forthcoming review by Schlesinger (*Advan. Virus Res.* **14**). Estimates of the size of virus morpho-

logical substructures have been calculated from these data in several ways and compared to figures from the literature. It has been assumed that when purified virus is labeled with multiple amino acids, the pattern of radioactivity in acrylamide gel reflects the relative mass of each of the constituent polypeptide chains.

The validity of any of the values is obviously a function of the accuracy of the determinations of percentage composition and molecular weight of the viral DNA and of the individual protein components. The values obtained in Fig. 9 nevertheless indicate the approximate distribution of molecules in the virion and its substructures, and direct attention to points for discussion and further study.

MORPHOLOGICAL SUBUNITS				POLYPEPTIDE SUBUNITS						
APPEARANCE	NAME	NUMBER PER VIRION[a]	MOLECULAR WEIGHT	COMPONENT[f]	PERCENT OF VIRION PROTEIN[d]	M W[d]	NUMBER PER SUBUNIT[j]		NUMBER PER VIRION[k]	
							Calc.	(Pred.)	Calc.	(Pred.)
VIRION	DNA		23,000,000[b]							
	PROTEIN		150,000,000[c]							
◯	HEXON	240	210,000[a] 400,000[e] 320,000[f] 360,000[g]	II	50.9	120,000	3	(6)	640	(1440)
(nine hexons)		20	3,600,000[h]	II (80%)[i] VIII,IX(20%)		120,000 13,000				
◯—	PENTON	12	280,000[a] 1,100,000	III,IV	8.6					
◯	PENTON BASE	12	210,000[a]	III	4.6	70,000	8	(5)	100	(60)
▭—	FIBER	12	70,000[a]	IV	4.0	62,000	8	(1)	100	(12)
CORE	DNA	1	23,000,000							
	PROTEIN		29,000,000[f]	V	5.4	44,000	180		180	
				VI,VII	14.2	24,000	890		890	
				VIII,IX	3.5	13,000			400	
				X	4.0	7,500			800	

FIG. 9. The polypeptide structure of the type 2 adenovirion.

[a] From Valentine and Periera (1965).

[b] From Green et al., 1967.

[c] Calculated from (b) and the value 0.133 for the proportion of DNA in purified virus (Green and Piña, 1964) as follows: MW protein = MW virion − MW$_{DNA}$ = $(23 \times 10^6)/(0.133) - (23 \times 10^6) = 150,000,000$.

[d] From Table 1 of Maizel et al. (1968). The values for percent of virion protein in the various components is the average of the distributions determined using mixed amino acid-labeled virus.

[e] From Petterson et al. (1967).

[f] Calculated from the percentage of virion protein in a given morphological subunit, the number of such subunits per virion, and the total mass of protein per virion: MW$_{morphological \ subunit}$ = (150×10^6) (percent protein/100)/(number of subunits). For example, MW$_{hexon}$ = $(150 \times 10^6) (0.51)/(240) = 320,000$.

[g] Calculated from the molecular weight and number of polypeptides per subunit, i.e., for hexons 3 × 120,000 = 360,000.

[h] Calculated from nine hexons of MW 400,000.

[i] It is speculated for the purpose of this table that the particles lacking DNA, sedimenting between free hexons and cores, and consisting of 80% hexon polypeptide (II) and 20% components VIII and/or IX (see Fig. 4) are the previously observed aggregates containing nine hexons (Smith et al., 1965; Russell et al., 1967).

[j] The calculated number of polypeptides per morphological subunit is the nearest integral number of the appropriate peptides per virion[k] divided by the number of morphological subunits per virion and expressed as the nearest whole number (e.g., 640 hexon peptides/240 morphological subunits = 2.7 ≡ 3 polypeptides/hexon). The predicted number (in parentheses) is based on virus morphology (Valentine and Pereira, 1965).

[k] The number of polypeptides per virion is calculated from the percent and molecular weight of a virion component as follows: Number of polypeptides = (150×10^6) (fraction of protein in a given component)/(MW of the component). The predicted number of polypeptides is the product of the predicted number of structure units per morphological unit[j] and the number of morphological subunits per virion.

From the known number of capsomeres per virion (Horne et al., 1959), it is possible to calculate an approximate molecular weight for some of the subviral structures. Peptide II is the major peptide of the virion and is the only constituent found in soluble hexons. Since this peptide makes up 51% of the total virion protein, and there are 240 hexons per virion, it can be calculated that the molecular weight of a single hexon should be approximately 320,000. This is reasonably close to the molecular weight of 400,000 determined by Petterson et al. (1967) and 310,000 determined by Köhler (1965) by sedimentation velocity and diffusion. Both figures, however, are considerably larger than the value of 210,000 calculated from measurements of hexons in electron micrographs (Valentine and Pereira, 1965). If, as would seem likely, the correct figure is somewhere between 310,000 and 400,000, and the molecular weight of peptide II is 120,000, there would be three polypeptide chains per soluble hexon. This is half the number expected from theoretical considerations based on the need for six identical sites to combine with the six neighboring hexons (Casper and Klug, 1962). It is possible, however, that a single polypeptide chain of this size could, when folded, have two equivalent combining sites. This difficulty could be avoided and the large size of the hexon peptide would be explained, if component II consisted of two identical polypeptide chains held together by covalent bonds other than disulfide linkage. [The presence of disulfide bonds appears to be ruled out by the use here of conditions adequate to reduce and separate other disulfide-linked polypeptides, e.g., γ-globulins (Scharff, 1967).] The possibility of two identical covalently linked chains can be resolved only by further chemical analysis. Studies reported in the following papers (White, Maizel, and Scharff; Horwitz, Maizel, and Scharff, in preparation) provide no evidence for a precursor of peptide II as would be expected if separate chains are synthesized and then joined. Both Smith et al. (1965) and Russell et al. (1967) have pointed out that electron micrographs of mildly disrupted virions commonly show aggregates of nine hexons. It is noteworthy that the surface of the virion could be constructed with one of these aggregates for each of the twenty faces of the icosahedron, leaving a group of five hexons surrounding a penton (Valentine and Pereira, 1965) at each vertex as has been illustrated in the model drawn in Fig. 9 (heavy outline). Large aggregates having component II, the hexon peptide, but sedimenting more rapidly than completely released hexons, have in fact been observed (Fig. 4); but they differ significantly from soluble hexons in containing peptides VIII and/or IX. Since the latter two peptides are poorly resolved, it is impossible to say whether one or both are associated with each group of hexons (Fig. 9, footnote i). Their presence suggests that they may serve to stabilize the structure.

The molecular weight calculated for the penton (1,100,000) is considerably higher than expected. To the degree that other unresolved proteins might be present in the III–IV peak of virions, this calculation would give a high figure. However, it is clear from experiments in which peptides III and IV were resolved (not shown) that there are approximately equal amounts of penton base and fiber peptides. If, as has been suggested (Valentine and Pereira, 1965), the penton base is similar in size to the hexon, one would expect the molecular weight of the whole penton to be between 640,000 and 800,000, a figure considerably higher than the 280,000 estimated from electron micrographs. The presence of equal amounts of penton base and fiber protein in the virion further suggests that both are made up of multiple identical polypeptide chains. On the basis of symmetry considerations, one might predict five peptides per penton base, and if there are equal amounts of penton and fiber a MW of 660,000 can be calculated for the whole penton using the molecular weights of III and IV. The number 5 is not in agreement with the value 8 calculated in Fig. 9. These discrepancies, too, will have to be resolved by further isolation and characterization. In these studies there has been,

ε yet, no evidence for a different peptide representing the terminal globular end of the fiber.

The peptides (V, VI, and VII), which are associated with the viral DNA in a nucleoprotein core, make up approximately 20% of the virion protein. They consist of at least three different polypeptide chains. These are relatively rich in basic amino acids (cf. Table 1, Maizel et al., 1968) and could account for the relatively higher content of those amino acids in virions (Polasa and Green, 1967) than in purified hexons (Biserte et al., 1964; Petterson et al., 1967). They are not histonelike, however, because they contain tryptophan. The observed requirement for arginine in the production of a factor necessary for virus maturation is of interest in this connection (Rouse and Schlesinger, 1967). Laver et al. (1967) and Russell and Knight (1967) have also presented evidence for internal proteins, and cores have been observed by Lawrence and Ginsberg (1967) and Philipson (1967) during uncoating.

While there may be large numbers of molecules of peptide X in virions, its location and role remain unknown.

The finding of multiple polypeptide chains in the various parts of the virions which differ in chemical and physical characteristics should not be surprising, since even the small, relatively simple, RNA viruses polio and EMC have proved to be more complex than originally predicted (Maizel, 1963; Rueckert and Duesberg, 1966). While some of these proteins serve to protect the viral nucleic acid and to promote interaction with the host cell, other functions may also exist. These could be related to maintaining compact viral structure, to the transport of viral DNA into the nucleus of the host cell, or even to the synthesis and control of viral macromolecules early in the replicative cycle.

If it is assumed that each gene is represented once and that only one strand of the DNA is translated, the total genome could code for about 1.3×10^6 daltons of unique amino acid sequence. The sum of the molecular weights of the virion peptides is approximately 3.8×10^5 daltons, or 29% of the genome. The viral DNA could code for approximately 20 additional peptides, which presumably function inside the infected cell. At least one additional antigen has been detected in cells transformed by tumorigenic strains, and also during lytic infection by nontumorigenic strain, but efforts to detect viral-specific enzymes have largely failed (Green, 1966). Thus, if in fact all the genome is translated into protein, and if there is no gene duplication, two-thirds of the protein products of the adenovirus genome remain to be discovered.

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13

The Polypeptides of Adenovirus

III. Synthesis in Infected Cells[1]

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Eight structural polypeptides of adenovirus type 2 can be identified in the infected HeLa cell. All are stable; none is a precursor of another. The various polypeptides are synthesized at very different rates, which do not reflect the ratio in which they are represented in the virion itself. For example, the penton-base and fiber polypeptides are made in considerable excess, whereas the three polypeptides comprising the inner viral core are in relatively short supply. At least 80% of the viral protein found in infected cells is in the form of free hexons, pentons, and fibers, the polypeptides of which have been identified.

Host protein synthesis proceeds completely normally until after viral capsid protein synthesis begins, then declines in parallel with the gradual rise in the rate of viral protein synthesis. If viral DNA replication is inhibited, no capsid proteins are made and no shutdown of cellular protein synthesis occurs; even when limited production of viral DNA and capsid protein is allowed, host cell protein synthesis is little affected even many hours later. This is consistent with the suggestion that the inhibition of cell protein synthesis may be attributed to competition for ribosomes between host and viral messenger RNA, rather than to any virus-coded "cell shutdown" protein.

The viral messenger RNA molecules coding for each of the several structural proteins have approximately the same stability. In the presence of actinomycin D, they decay with a mean half-life of 6 hours.

INTRODUCTION

In two earlier papers (Maizel *et al.*, 1968a, b) methods were described for the dissociation of purified adenoviruses to their constituent polypeptides and for the separation of these molecules on acrylamide gels. At least nine distinct polypeptides were identified. By examining the composi-

tion of purified soluble antigens, artificially produced cores, and naturally occurring "top components," it was possible to determine the location in the virion of eight of the nine peptides. Three are present in the outer capsid: the "hexon" polypeptide of molecular weight (MW) 120,000, the "penton-base" polypeptide of MW 70,000, and its associated "fiber" polypeptide of MW 62,000. Two minor peptides of MW 13,000 are intimately associated with hexons. Three others of MW 44,000, 24,000, and 24,000, comprising some 20% of the total viral protein, are arginine-rich peptides making up an inner core. The present paper deals with the synthesis of these proteins in infected cells.

MATERIALS AND METHODS

Most of the Materials and Methods used in this work, including the purification and labeling of virus, the handling of "spinner cultures," polyacrylamide gel electrophoresis, and DEAE-cellulose chromatography, have been described in detail in two earlier papers (Maizel et al., 1968a, b).

Infection and labeling of cells. HeLa S3 cells growing exponentially in suspension culture were washed three times in serum-free medium, and resuspended at a concentration of 10^7 cells per milliliter in Puck's saline (Marcus et al., 1956) or Eagle's Minimum Essential Medium (MEM) (Eagle, 1959). Purified adenovirus type 2 was then added at a multiplicity of 300–600 particles per cell, and the culture was gently stirred for 15 min at room temperature; under these conditions approximately 30 % of the particles were adsorbed (Dr. John Daniels, unpublished). The infected cells were then diluted to a concentration of 1.5 × 10^5 cells per milliliter with prewarmed Eagle's MEM for suspension culture, supplemented with 5 % calf serum, and incubated at 37° in suspension culture. In those experiments in which the infected cells were to be labeled with radioactive valine and threonine, the medium contained valine and threonine at one-tenth the normal concentration, preliminary experiments having shown that the yield of adenoviral proteins and particles was unaffected by these conditions. Pulse-labeling was carried out by transfering 10 ml of the infected cell suspension to a tube containing 1–10 μC each of ^{14}C-labeled valine and threonine. Pulses were terminated by adding a large excess of ice-cold Earle's saline, or chased by adding valine and threonine to a final concentration 10 times that of normal Eagle's MEM. At the conclusion of the experiment the cells were washed in ice-cold Earle's saline.

Analysis of infected cells. For sucrose gradient analysis, washed cells were resuspended in a small volume of 0.01 M Tris-HCl buffer pH 8.1, disrupted by agitation for 60 sec at 4° in a Mullard sonic disintegrator, and treated with 0.5 % sodium deoxycholate. The resulting lysate was sedimented on a linear sucrose gradient, which was then analyzed for trichloroacetic acid (TCA)-precipitable radioactivity and UV absorbance at 280 mμ as previously described (Maizel et al., 1968a).

Cell lysates or fractions recovered from sucrose gradients were dissociated for acrylamide gel electrophoresis by adding sodium dodecyl sulfate (SDS) and 2-mercaptoethanol (ME) to final concentrations of 2 % and 1 %, respectively. [Urea (0.5 M) was also added in the earlier experiments, but makes no difference to the final result.] After incubation for 30 min at 37°, or 1 min at 100° (Maizel et al., 1968a), the samples were dialyzed overnight against 0.1 % SDS, 0.1 % ME in 0.01 M phosphate buffer pH 7.2. Acrylamide gel electrophoresis was carried out as previously described (Maizel et al., 1968a).

Serological procedures. Three rabbit antisera were used to characterize the viral proteins recovered from DEAE columns or sucrose gradients. (i) A broadly reacting antiserum ("R 47") was produced by successive injections of whole purified adenovirus type 2 dialyzed free of cesium chloride, of virus which had been dissociated with alkali (Maizel et al., 1968a), and of virus dissociated wtih SDS, urea, and ME (Maizel et al., 1968a). In all cases the inoculum was emulsified in complete Freund's adjuvant (Difco) containing 4 mg/ml of *Mycobacterium butyricum* (Difco), and injected either into the foot pads or intramuscularly. (ii) A "type-specific" antiserum ("R 47-absorbed" (R-47 ab)) was made by repeatedly incubating antiserum R 47 with both intact and alkali-treated adenovirus type 5 until there was no longer any detectable precipitate. Complete absorption was demonstrated by the inability of "R 47-ab" to precipitate any radioactivity from labeled lysates of cells infected with adenovirus type 5, and by the presence in the antiserum of excess type 5 antigens detectable by agar diffusion. (iii) An antiserum "R 52," reacting specifically with pentons was produced by immunizing rabbits with a protein that had been prepared by preparative acrylamide gel electrophoresis of SDS-treated adenovirus type 2

(Maizel, 1964) and did not react with any of the standard group or type-specific antisera.

Gel diffusion and indirect radioisotope precipitation were carried out as described previously (Scharff and Levintow, 1963).

RESULTS

Identification of Viral Peptides in Infected Cells

Adenovirus type 2-infected HeLa cells, labeled with valine-^{14}C and threonine-^{14}C from 10.5–24 hours, were mixed with highly purified valine-^{3}H and threonine-^{3}H-labeled virions, then dissociated with SDS and ME, and analyzed by acrylamide gel electrophoresis (Fig. 1A). Uninfected cells (Fig. 1B) were mock-infected, labeled, and treated in the same way as the infected culture.

All the viral structural peptides were clearly recognizable in the infected cell. Roman numerals have been assigned to each of those previously identified (Maizel *et al.*, 1968a,b). Component II is the polypeptide comprising the hexon, III the penton base, and IV the fiber, the latter two migrating at closely similar rates. Peptides V, VI, and VII were shown to be arginine-rich core proteins, and VIII and IX were found in association with aggregates of hexons (Maizel *et al.*, 1968b).

Since all the viral peptides contain approximately the same relative proportions of valine and threonine (Maizel *et al.*, 1968a), the area under each peak, above the background of cell proteins, represents the approximate amount of the peptide present. Dividing each of these areas by the molecular weight of the corresponding peptide (Maizel *et al.*, 1968a) provides a measure of the relative numbers of molecules of each. It becomes apparent that the infected cell synthesizes some viral peptides in greater numbers than others. For example, there are five times as many molecules of peptide II as there are of peptide V. Nor are the peptides present in the same ratio in which they occur in the virion. This is especially evident for the penton-base and fiber peptides (III, IV), which are present in the infected cell in considerably greater excess than, for in-
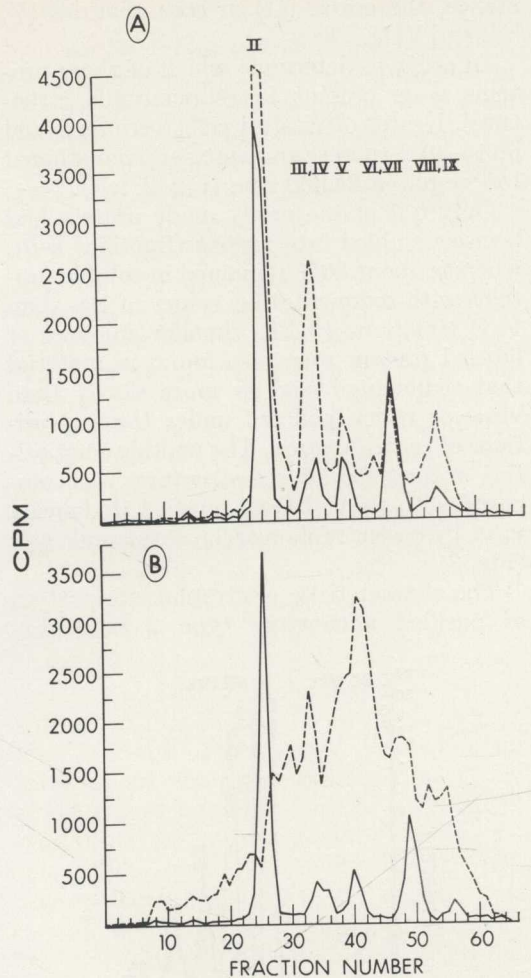

Fig. 1. Acrylamide gel electrophoresis of dissociated adenovirus-infected cells. HeLa S3 cells, infected with adenovirus type 2 (Fig. 1A) or uninfected (Fig. 1B), were labeled with valine-^{14}C and threonine-^{14}C from 10.5–24 hours later. The cells were then washed, and mixed with ^{3}H-valine and threonine-^{3}H labeled purified adenovirus type 2. After treatment with SDS and ME, followed by overnight dialysis, the peptides were separated by electrophoresis for 5.5 hours at 120 V on a 5% polyacrylamide gel containing 0.1% SDS, pH 7.2. The ^{14}C-labeled cell lysate is shown as a dashed line; the ^{3}H-labeled purified adenovirus as a solid line. The origin (cathode) is on the left and the anode on the right of this and all subsequent electropherograms. Roman numerals have been assigned to the viral structural polypeptides identified in the preceding paper (Maizel *et al.*, 1968b).

stance, the hexon (II) or core peptides (V, VI, and VII).

In order to determine which of these proteins were present in sedimentable structures, lysates of washed cells were analyzed on 15–30 % sucrose gradients, and centrifuged for 85 min at 24,000 rpm (Fig. 2, left).

Only 6 % of the newly made protein had been assembled into virions (fractions 5–9), whereas about 80 % remained in soluble proteins with sedimentation values of less than 15 S (fractions 18–24). Smaller amounts of labeled protein were also found in material that sedimented slightly more slowly than virus or which pelleted under these conditions of centrifugation. The peptide composition of these ^{14}C-labeled structures was compared with that of CsCl-purified ^{3}H-labeled virus by coelectrophoresis on acrylamide gels (Fig. 3).

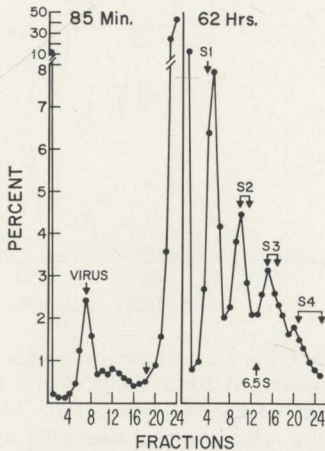The characteristic electrophoretic pattern of purified adenovirus type 2 is readily
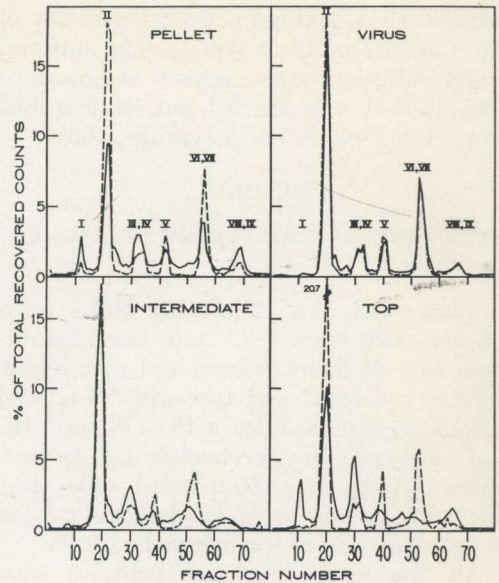

Fig. 3. Acrylamide gel electrophoresis of fractions from a sucrose gradient of a lysate of adenovirus-infected cells. The following fractions were taken from the 85-min, 15–30% sucrose gradient shown on the left of Fig. 2: pellet (fraction 1), virus (fraction 7), intermediate material (fractions 10–14) and material from the top of the gradient (fractions 20–24). Each sample was mixed with valine-^{3}H and threonine-^{3}H labeled adenovirus type 2, highly purified by repeated fluorocarbon extraction and cesium chloride equilibrium gradient centrifugation. Each mixture was then dialyzed, dissociated with SDS, ME, and urea, dialyzed again, and subjected to electrophoresis in a 5% acrylamide gel for 5.5 hours at 120 V. Radioactivity due to ^{14}C is plotted as a solid line, that due to ^{3}H as a dashed line, as a percentage of the total counts of the corresponding type recovered from the gel. The figures have been normalized to make the total number of ^{14}C and ^{3}H counts on each gel equal. The origin (cathode) is to the left, anode to the right in these and all subsequent electropherograms.

Fig. 2. Rate-zonal centrifugation of dissociated adenovirus-infected cells. HeLa S3 cells infected with adenovirus type 2 were labeled with valine-^{14}C and threonine-^{14}C from 16 to 24 hours postinfection. The cells were then lysed, and spun at 24,000 rpm on a 15–30% linear sucrose gradient for 85 min (left panel), or a 5–20% gradient (containing rabbit γ-globulin (6.5 S) as an optical density marker) for 62 hours (right panel). Timed fractions were collected (fraction 1 being the deposit at the bottom of the tube, and fraction 25 the top of the gradient), precipitated with TCA, and collected on Millipore filters for counting. The radioactivity of each fraction is plotted as a percentage of the total number of counts layered onto the gradient.

recognized in the top right panel (Fig. 3), where virus isolated from the sucrose gradient is compared with reference virions purified by equilibrium centrifugation. The pelleted material showed the same general electrophoretic pattern as purified virus except that there was a significantly higher "background" throughout the gel. The particles of intermediate sedimentation constant (sucrose gradient fractions 10–16)

differed from pure virus in that they were relatively deficient in the core peptides (V, VI, and VII). In this respect they resemble the "top component" from CsCl density gradients (Maizel et al., 1968b), and probably represent "empty" virions. The proteins isolated from the top of the sucrose gradient (fractions 18–24) were also deficient in core peptides, but contained relatively more of the penton and fiber peptides than pure virus (see also Fig. 1).

These proteins found at the top of the sucrose gradient could themselves be resolved into three major peaks by more prolonged centrifugation (Fig. 2, 62 hours). Based on a rabbit γ-globulin marker (6.5 S), the three components, S1, S2, and S3 were calculated to have sedimentation constants of 11 S, 8 S, and 5.5 S, respectively. Very little protein remained at the top of the gradient (S4).

It seemed likely that the three small entities, S1, S2, and S3, separated from infected cell lysates by sucrose gradient centrifugation corresponded with the three known morphologically identifiable subunits separated by DEAE-cellulose chromatography (Wilcox et al., 1963; Valentine and Pereira, 1965). To test this assumption, free hexons, pentons, and fibers from infected cells were purified by DEAE-cellulose chromatography (Maizel et al., 1968b) and submitted to rate-zonal centrifugation in parallel with the original cell lysate (Fig. 4).

The 11 S structure (S1) was found to cosediment with DEAE-cellulose-purified hexons, the 8 S structure (S2) with pentons, and the 5.5 S structure (S3) with fibers.

Antigenic characterization of the three subunits S1, S2, and S3 confirmed their identity as hexons, pentons, and fibers, respectively (Table 1).

An antiserum made against purified degraded adenovirus type 2 (antiserum "R 47") formed precipitin lines in Ouchterlony gel diffusion against DEAE-cellulose-purified hexons, pentons, or fibers. By contrast, the type-specific antiserum ("R 47 absorbed") reacted only with pentons and fibers, while antiserum "R 52" reacted only with pentons. Clearly, pentons carry at least two antigenic determinants, one of which is type specific,

and the other not present on fibers. When the same antisera were tested for their ability to precipitate the three radioactively labeled subunits from sucrose gradients, it was found that "R47" precipitated more than 95% of the radioactivity in S1, S2, or S3, whereas "R47 ab" precipitated S2 and S3, and "R52" precipitated only S2. The data confirm that S1, S2, and S3 represent the hexon, penton, and fiber, respectively.

The polypeptide composition of each of the three viral subunits was then determined by acrylamide gel electrophoresis. The peak fractions from S1, S2, and S3 were rebanded on sucrose gradients, dissociated with SDS, and examined on acrylamide gels in parallel with the starting material (infected cells) and the soluble proteins from the top of the gradient (S4) (Fig. 5).
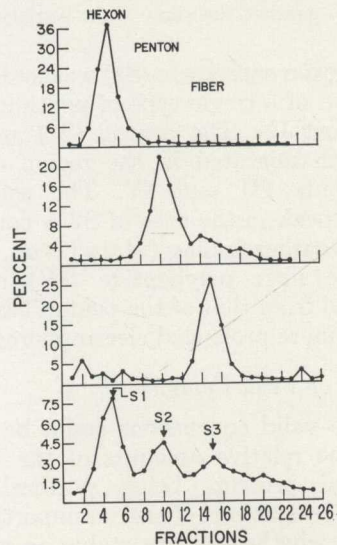

FIG. 4. Rate-zonal centrifugation of viral subunits separated by DEAE-cellulose chromatography. Hexons, pentons, and fibers were purified from valine-^{14}C and threonine-^{14}C labeled adenovirus 2-infected HeLa cells by DEAE-cellulose chromatography. Each type of subunit was then spun in a 5–20% sucrose gradient at 24,000 rpm for 62 hours together with the original infected cell lysate shown in Fig. 2 (right panel). Timed fractions were precipitated with TCA, collected onto Millipore filters, and counted. Radioactivity is expressed as a percentage of the total counts placed on the gradient. The top of each gradient is on the right.

TABLE 1

SEROLOGICAL IDENTIFICATION OF
VIRAL SUBUNITS[a]

Subunit	Antiserum		
	R47 Broadly reacting	R47-ab Type-specific	R52 Antipenton
Hexon	+	−	−
Penton	+	+	+
Fiber	+	+	−
S1	+	−	−
S2	+	+	+
S3	+	+	−

[a] Hexons, pentons, and fibers, separated from infected cells by DEAE-cellulose chromatography, were tested by gel diffusion against three antisera. The fractions S1, S2, and S3 from a sucrose gradient performed on the same cell lysate were tested by indirect radioisotope precipitation against the same three antisera.

The hexon capsomere (S1) was found to be composed of a single type of peptide (virion component II). The penton (S2) and fiber (S3) both migrated in the region of viral components III and IV. The somewhat broader peak in the case of S2 is consistent with the earlier finding (Maizel *et al.*, 1968b) that the fiber polypeptide (IV) and be separated from that of the penton base (III) only by more prolonged electrophoresis.

Stability of Viral Peptides

Before valid conclusions could be drawn about the relative amounts of the various structural proteins being synthesized in infected cells, it was of crucial importance to discover whether all are stable or whether some are precursors of others. Accordingly, cells actively synthesizing viral structural proteins 16 hours postinfection were pulse-labeled for 10 min, then chased with a 200-fold excess of unlabeled amino acids to allow protein synthesis to continue in the absence of further incorporation of isotope. Cells taken immediately after the commencement of the chase were compared with those taken 8 hours later, by acrylamide gel electrophoresis (Fig. 6).

The effectiveness of the chase was demonstrated by the fact that no detectable in-

crease in TCA-precipitable radioactivity occurred during the 8-hour period. All the peptides synthesized during the pulse were present in much the same amount 8 hours later. Similar results were obtained when infected cells were pulse-labeled at 12 or 22 hours, or when the pulse was as short as 2 min. In the latter case, however, results were somewhat more difficult to interpret because of the presence of incomplete peptides on ribosomes at the termination of the pulse, and their subsequent completion during the first 2–4 min of the chase. Nevertheless, there was no evidence for the existence of precursor peptides that are subsequently cleaved or united into smaller or larger molecules,
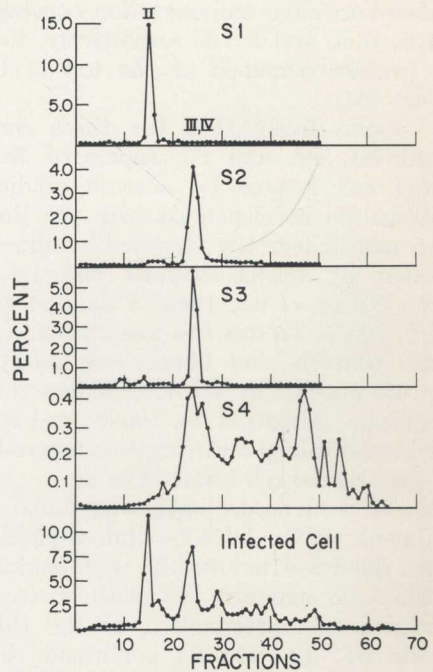

FIG. 5. Acrylamide gel electrophoresis of viral subunits separated by rate-zonal centrifugation. Material from the peak fraction of S1, S2, and S3, respectively, was further purified by a second sucrose gradient centrifugation. The peak fraction from each of these runs was taken, as was the soluble protein (S4) from the top of the original gradient, and also an unfractionated infected cell lysate. All were treated with SDS, ME, and urea and subjected to acrylamide gel electrophoresis. Radioactivity is plotted as a percentage of the total counts present on the original sucrose gradient.

respectively (Summers and Maizel, 1968). Clearly, all the structural proteins of adenovirus type 2 are stable.

Time Course of Synthesis of Viral Peptides

At various times after infection, cells were pulse-labeled for 30 min with valine-^{14}C and threonine-^{14}C, then dissociated and analyzed on acrylamide gels (Fig. 7).

Uninfected HeLa cells synthesize so many different proteins that their electropherogram appears as a continuous smear with occasional peaks superimposed on the background. Infected cells presented the same picture until 9–10 hours after infection, after which distinct peaks corresponding to the structural proteins of the virus became recognizable. The rate of synthesis of these viral peptides increased until 16–19 hours

postinfection, when it reached a maximum which was maintained for a further 6–10 hours before gradually declining. As infection progressed there were no striking changes in the rate of synthesis of viral peptides relative to one another. One possible exception was the peak immediately to the right of the hexon peptide (II), which was relatively conspicuous early but did not increase significantly thereafter. This appears to be a very minor peptide, not always detected; its nature has not yet been determined.

Shutdown of Host Protein Synthesis

It is clear from Fig. 7 that host protein synthesis was totally unaffected until at least 10 hours postinfection, when the synthesis of viral structural proteins first became detectable. Even for some time thereafter, the synthesis of viral protein was
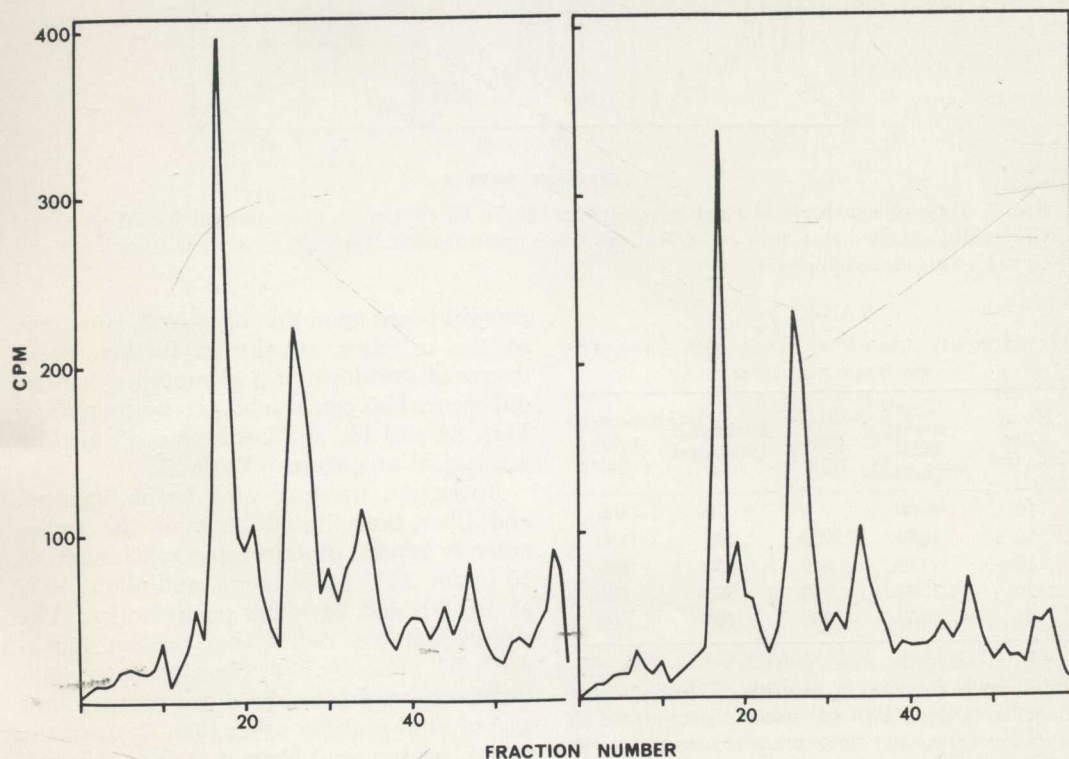

Fig. 6. Stability of viral polypeptides. HeLa S3 cells infected 16 hours earlier with adenovirus type 2 were pulse-labeled for 10 min with 30 μCi of valine-^{14}C and threonine-^{14}C. Half the culture was immediately chilled to stop further incorporation. The other half was "chased" for a further 8 hours by the addition of a 200-fold excess of unlabeled valine and threonine. Both cultures were washed, spun down, treated with SDS, ME, and urea, and analyzed by gel electrophoresis. The unchased culture is shown on the left, the cased on the right.
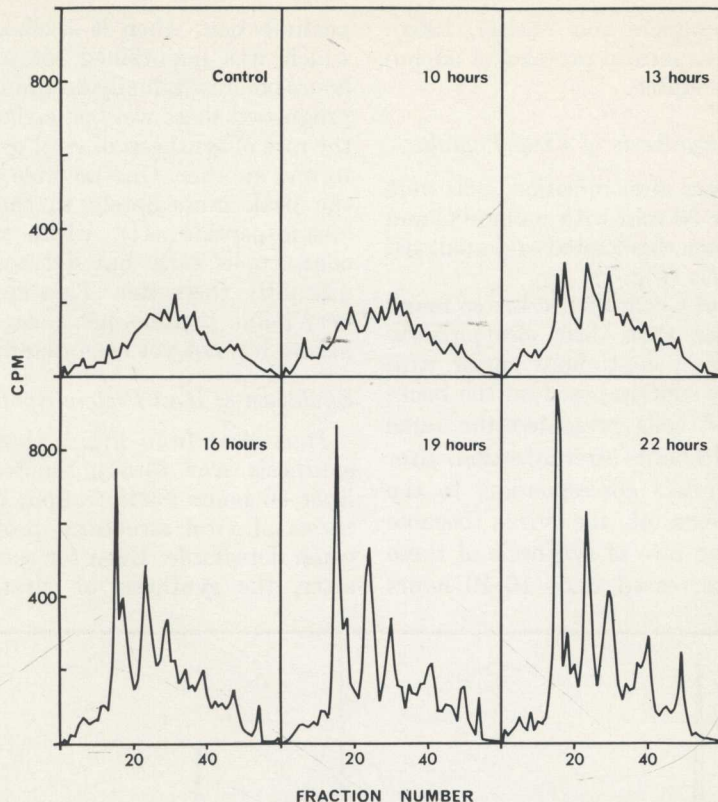
Fig. 7. Rate of synthesis of viral polypeptides. HeLa S3 cells were pulse-labeled for 30 min with 5 μCi of valine-^{14}C and threonine-^{14}C at various times postinfection. The cells were then dissociated and analyzed by gel electrophoresis.

TABLE 2

RELATIONSHIP BETWEEN VIRAL AND CELLULAR PROTEIN SYNTHESIS[a]

Hours after infection	Total counts TCA-precipitable	R47-precipitable	Estimated total viral	Estimated total cellular
10	1400	0	0	1400
12	1450	220	290	1160
16	1740	640	850	890
20	1580	720	960	620
24	1350	560	750	600

[a] Infected cells were pulse-labeled with ^{14}C-amino acids for 30 min at each of the indicated times. Antiserum R47 was used to precipitate all hexon, penton, and fiber protein present in the cell lysate at each time. The total viral protein newly synthesized during each pulse was estimated from the fact that these three make up approximately 75% of the total viral protein in electropherograms of infected cells. The quantity of cellular protein synthesized during the same interval was calculated by subtracting the latter figure from the total TCA-precipitable counts.

superimposed upon that of the cell. However, as the infection progressed further, some degree of shutdown of host protein synthesis did occur. This can also be seen by comparing Figs. 1A and 1B, and has been confirmed by serological procedures (Table 2).

Antiserum reacting with hexon, penton, and fiber precipitated none of the radioactively labeled protein being synthesized at 10 hours, 15% at 12 hours, and about 40% at 16, 20, and 24 hours postinfection. The nonprecipitable radioactive protein meanwhile fell away by half. Since the latter material consisted not only of cell proteins, but also of viral proteins other than those of the hexon, penton, and fiber, it is clear that cell protein synthesis was shut down by somewhat more than 50% by 20–24 hours postinfection.

These experiments seemed to indicate that the parental adenoviral genome does not code for any protein leading directly to the shutdown of cellular protein synthesis. This

was tested directly in the following way. At 6 or 10 hours postinfection infected cells received 5-fluoro-2-deoxyuridine (FUdR), 3×10^{-5} M, to inhibit DNA synthesis. Proteins were labeled with ^{14}C-amino acids from 20 to 30 hours, at which time half the cells were analyzed by precipitation with the broadly reacting adenoviral antiserum "R47" and the other half by electrophoresis (Fig. 8).

Following the addition of FUdR at 6 hours, no adenoviral structural proteins were demonstrable by either method, and the electropherograms were quantitatively as well as qualitatively indistinguishable from those of FUdR-treated uninfected cells. Hence it is clear that, in the absence of viral DNA replication, cell protein synthesis was unaffected until a day later. Even if FUdR was added as late as 10 hours postinfection, when viral DNA replication was well underway (Hodge and Scharff, in preparation),

host protein synthesis was still relatively unimpeded at 20–30 hours despite the production of considerable amounts of viral structural proteins.

Decay of Viral Messenger RNA

Since the major viral structural peptides were not made in equimolar amounts, it was important to determine that this unbalanced synthesis was not due to differences in stability of the respective messenger RNA molecules. Therefore, cells synthesizing viral structural proteins at maximum rate were treated with actinomycin D (1 or 10 μg/ml), and at intervals thereafter were pulse-labeled with valine-^{14}C and threonine-^{14}C, then analyzed by electrophoresis. Untreated infected cultures pulse-labeled at the same times served as controls (Fig. 9).

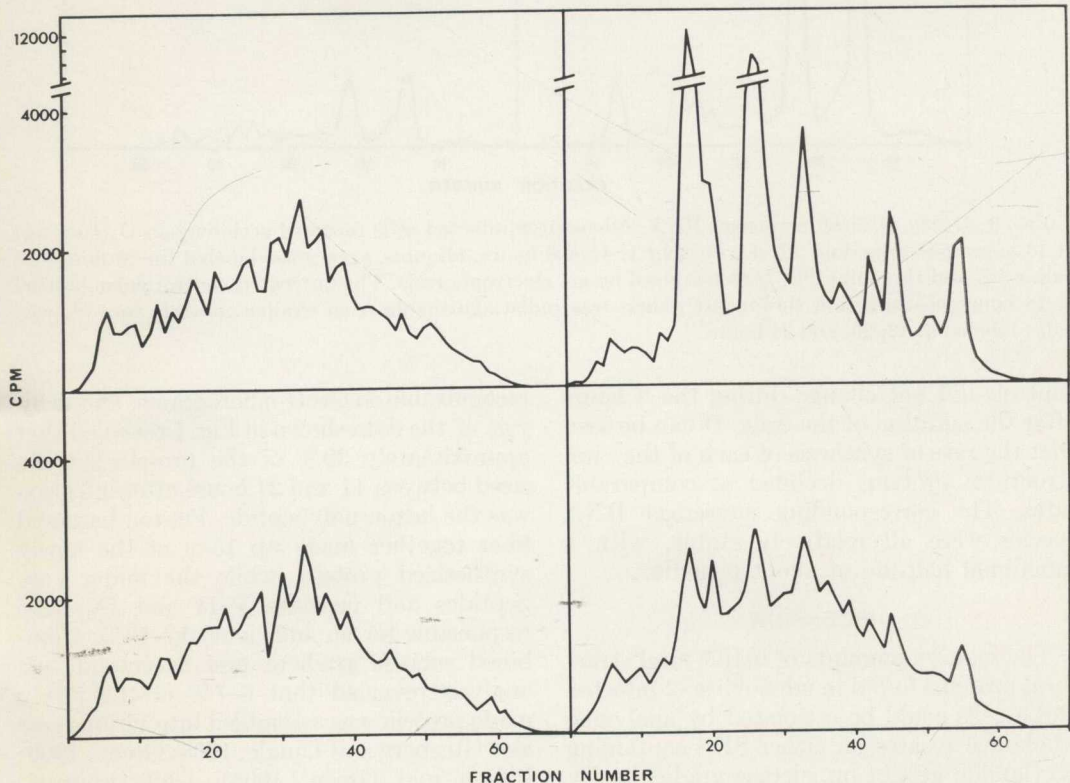Results were similar with either dose of actinomycin, and the appearance of the

FIG. 8. Protein synthesis in the presence of 5-fluoro-2-deoxyuridine (FUdR). Adenovirus-infected HeLa cells were labeled with valine-^{14}C and threonine-^{14}C from 20–30 hours postinfection, having received 3×10^{-5} M FUdR at 6 hours (lower left panel), 10 hours (lower right), or not at all (upper right.) An uninfected control (upper left) received FUdR at 6 hours and was similarly labeled with valine-^{14}C and threonine-^{14}C at 20–30 hours. All cultures were then analyzed by acrylamide gel electrophoresis.
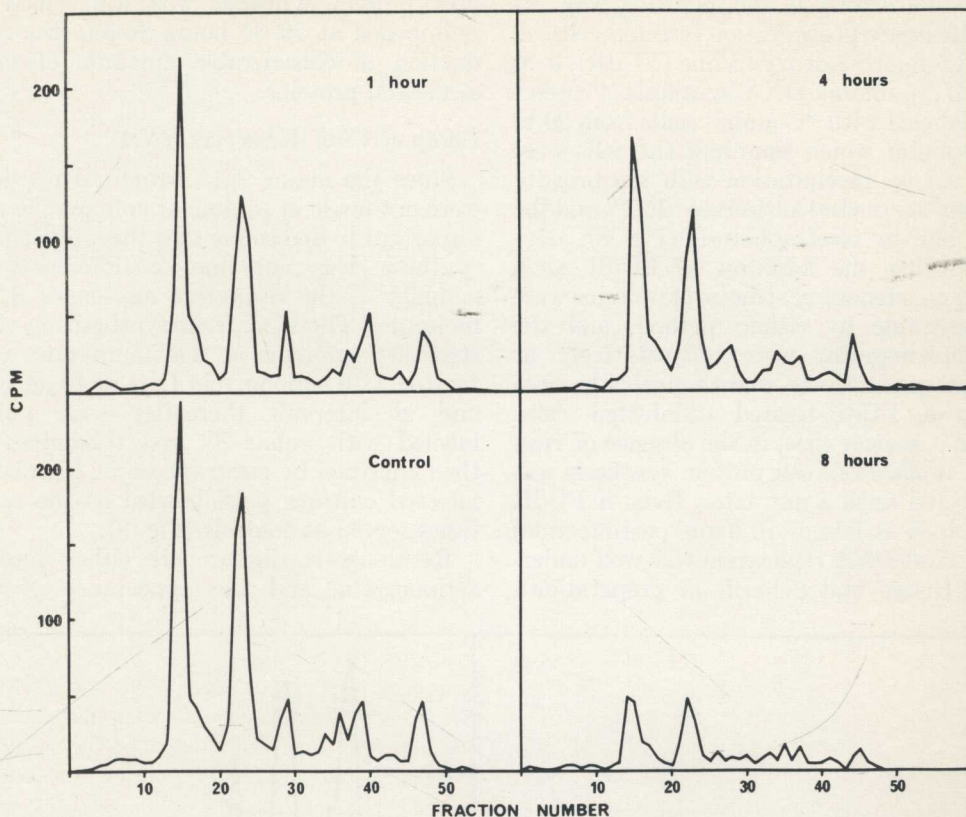
FIG. 9. Decay of viral messenger RNA. Adenovirus-infected cells received actinomycin D (1 μg/ml) at 16 hours postinfection. After a further 1, 4, or 8 hours, aliquots were pulse-labeled for 30 min with valine-14C and threonine-14C, then analyzed by gel electrophoresis. The untreated control pulse-labeled at 16 hours postinfection (lower left panel) was indistinguishable from similar controls (not shown) pulse-labeled at 17, 20, and 24 hours.

controls did not change during the 8 hours after the addition of the drug. It can be seen that the rate of synthesis of each of the viral structural proteins declined at comparable rates. The corresponding messenger RNA species were all relatively stable, with a functional half-life of about 6 hours.

DISCUSSION

The relative amounts of major viral structural proteins found in adenovirus-2-infected HeLa cells could be estimated by analyzing whole cell lysates on either SDS-containing acrylamide gels or on sucrose gradients. By either method the major surface and internal proteins found in the virion accounted for 50–60% of the protein made during the latter part of the infectious cycle. All the major polypeptides found in the virion were readily recognizable on electropherograms, and analysis of the data shown in Fig. 1 revealed that approximately 30% of the protein synthesized between 11 and 24 hours after infection was the hexon polypeptide. Penton base and fiber together made up 15% of the newly synthesized protein, while the major core peptides and peptides VIII and IX were responsible for an additional 12–13%. Combined sucrose gradient and acrylamide gel analysis revealed that 6–7% of the newly made protein was assembled into virions (see also Ginsberg and Dingle, 1965; Green, 1966; Polasa and Green, 1965), while approximately 22% existed as free hexons, 13% as pentons, and 12% as fibers (see also Wilcox and Ginsberg, 1963; Wilcox et al., 1963). An approximately 6-fold excess of hexon peptide was synthesized compared to that

which was assembled in the virion, while the penton base and fiber peptides were made in approximately 20-fold excess. There was a 3- to 4-fold excess of core peptides.

All the structural viral polypeptides were shown to be stable. There was no evidence that any large peptide is subsequently cleaved into smaller ones as happens with the polioviral polypeptides (Summers and Maizel, 1968). Hence, it proved practicable to investigate the rates of synthesis of all these peptides throughout the multiplication cycle.

The various structural peptides are synthesized at different rates. There are a number of ways in which this might be brought about. The cistrons for some peptides could be represented several times in the viral DNA molecule, or control mechanisms could operate at the level of transcription or translation of viral messenger RNA. Equally puzzling is the finding that the viral peptides are not synthesized in the same ratio as they occur in the virion. The seemingly wasteful overproduction of subunits, particularly pentons and fibers, may relate to topographical difficulties in assembling proteins made in the cytoplasm into virions in the nucleus, to problems imposed by the assembly of a complex icosahedron, to nonstructural functions of some of the proteins (Levine and Ginsberg, 1967, 1968), or merely to the fact that the synthesis of individual peptides is not coordinated with the structural requirements of the virus.

In the absence of viral DNA replication, no capsid proteins were detectable by serological precipitation or acrylamide gel electrophoresis, even when cells were infected at very high multiplicity and when up to 20 hours were allowed for such proteins to accumulate in the presence of FUdR. This confirms the findings of Bello and Ginsberg (1967), and indicates that the information for these proteins encoded in the parental viral genome is repressed in some completely unknown way until after the viral DNA has replicated.

Host protein synthesis continues unabated until well after the commencement of synthesis of viral DNA and "late" proteins. When FUdR was added at a time when 10–20% of the final complement of viral DNA had already been made, the cells continued to synthesize host proteins at nearly normal rates even a day later. It can be concluded that neither parental nor progeny viral DNA codes for any molecule leading to the rapid or pronounced shutdown of host protein synthesis. Only in the second half of the cycle does the rate of synthesis of cellular proteins gradually diminish in parallel with a corresponding rise in the rate of synthesis of viral structural proteins, until finally 60% or more of the protein being made in the infected cell is viral. It seems unlikely that fiber antigen, which has been shown to inhibit macromolecular synthesis in uninfected cells (Levine and Ginsberg, 1967), causes the decrease in host cell protein synthesis since fiber is equally effective against both host and viral protein synthesis. As has been suggested by others (Levine and Ginsberg, 1967; Bello and Ginsberg, 1967), the inhibition of host protein synthesis could result from simple competition for free ribosomes between host and viral messenger RNA. After viral DNA replication begins, the rate of viral messenger RNA synthesis gradually increases, while that of cellular messenger RNA remains relatively unchanged for some time, then slowly declines until most of the new RNA being made is viral (Rose et al., 1965; Fujinaga and Green, 1966; Ginsberg et al., 1967). There is a progressively increasing probability that any given ribosome will attach to a viral rather than a host messenger RNA molecule. This gradual decline in the rate of host cell protein synthesis, beginning midway through the cycle, is not to be confused with the decline of host and viral protein synthesis that occurs toward the very end of the cycle (Bello and Ginsberg, 1967; Ginsberg et al., 1967).

The resolution provided by acrylamide gel electrophoresis in the neutral SDS system facilitated an investigation into the rate of loss of function of the mRNA's for each of the viral structural peptides. The mRNA's for all these peptides proved to be stable, with a mean functional half-life of about 6 hours.

ACKNOWLEDGMENTS

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