THE MULTIPLICATION OF INFLUENZA VIRUS

by

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STATEMENT

The experimental work reported in Section I of this thesis represents a joint investigation, and was carried out in close collaboration with Dr. H.J.F. Cairns.

The work reported in Section II was carried out entirely by myself. The study began with the suggestion by Dr. Cairns that an opportunity existed to develop an improved experimental system in which to study the quantitative aspects of influenza virus multiplication.

Richard Barry.
The work reported in this thesis was carried out in the Department of Microbiology, John Curtin School of Medical Research, Australian National University. I am indebted to Professor F.J. Fenner for the opportunity to undertake this work and for his constant help and encouragement.

To my supervisor, Dr. H.J.F. Cairns, I owe a particular debt of gratitude, not only for practical suggestions which have greatly aided the course of this work, but for many hours of fruitful discussion. Many of the ideas which are developed in this thesis are the outcome of these discussions.

I also wish to thank my friends of this Department, all of whom at some stage or another have provided advice or assistance.

Finally, I am indebted to Miss Margaret Briggs for the preparation of electron micrographs.
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PREFACE
This thesis has been divided into two sections. One section is concerned with the physical nature of influenza virus, and the other with the manner in which the virus multiplies.

The first and smaller of these sections deals with the relation between the physical entity of the virus and its biological characteristics. It is known that there are about ten times as many visible particles present in influenza virus preparations as can be detected by the normal biological tests of haemagglutination or infectivity determination. This excess may be due to the existence of classes of particles of differing biological activity, or to deficiencies in the methods of biological assay, or, for that matter, to both. The existence of this excess is of considerable importance in biological studies. As a preliminary to the biological studies which constitute the major part of this thesis, this problem was explored. The technique employed was that of equilibrium sedimentation in density gradients of low molecular weight solutes, introduced by Meselson, Stahl and Vinograd (1957), which offered a possible means of distinguishing differences in biological activity on a basis of particle density. Both standard and incomplete virus preparations have been examined.

The second and larger section deals with the biology of influenza virus, the aim being to provide a general explanation for the various manifestations of virus multiplication. At the
time these studies were begun, the need for a suitable host cell system for the quantitative investigation of influenza virus multiplication was apparent. Much of the confusion which dogs the literature of the more contentious aspects of influenza virus multiplication stems from the inadequacy of the whole egg as an experimental system. So, a reliable and easily handled experimental system had to be developed. Such a system was then used to investigate the nature of virus multiplication.

This section has been subdivided into three chapters as a convenient means of considering in order first, the multiplication of normal virus in normal cells, secondly, the multiplication of inactivated virus in normal cells, and finally, the multiplication of both active and inactive virus in interfered cells. These aspects of virus multiplication have considerable bearing on some of the most interesting and controversial facets of influenza virus reproduction, namely, the formation of incomplete virus, the nature of genetic recombination, and the process of interference.

On the basis of these studies, the various manifestations of virus multiplication have been classified into one of two classes. They can be regarded as being due either to the presence within cells of single, effective parental particles, or to the simultaneous presence and interaction within cells of more than one parental particle. These classes can be readily distinguished,
and have been called respectively, simple and co-operative forms of multiplication. Much more work is needed, however, to establish the general validity of this distinction, for any interpretation based on the existing evidence must of necessity be rather speculative. Nevertheless, the notion of co-operation, which is introduced in this thesis, although possibly oversimplifying the situation, offers a plausible interpretation of certain hitherto unrelated features of influenza virus multiplication.
SECTION I

THE PHYSICAL NATURE OF INFLUENZA VIRUS

Equilibrium Sedimentation in Density Gradients
INTRODUCTION

Influenza virus preparations always contain more morphologically characteristic particles, as determined by electron microscope count, than can be detected by the conventional infectivity or haemagglutination titrations for virus activity (Donald and Isaacs, 1954a). The relatively constant proportionality which is found to exist between the total number of particles present and biological activity could possibly be totally ascribed to inherent shortcomings in the methods of biological assay (cf. Fazekas de St. Groth and White, 1956b). On the other hand, there is some morphological variation in influenza virus preparations which suggests that perhaps all the forms present may not represent biologically active virus. In fact, it becomes important to decide whether this discrepancy is attributable wholly to deficiencies in the method of assay, or whether much of the virus is inactive. The analysis of influenza virus preparations by the technique of equilibrium sedimentation in density gradients (Meselson, Stahl and Vinograd, 1957) seemed a likely means of determining whether any functionally imperfect forms exist which may contribute to the observed deficiency. This provides a means of determining the density homogeneity of a virus population. Obviously, if potentially active particles differ from inactive or defective forms in particle density, this technique offers a ready means of fractionating these elements. This
section records an investigation of the physical nature of several well studied strains of influenza A virus with this technique. What is already known of the physical nature of influenza virus will be briefly considered before the behaviour of the virus in these density gradients is described.

The earliest studies of the physical nature of influenza virus were aimed primarily at determining the size of the virus particle. By ultrafiltration through collodion membranes, Elford, Andrewes and Tang (1936) obtained estimates of particle diameter in the range 80-120 m\(\mu\). This was narrowed to 87-99 m\(\mu\) by high speed centrifugation studies (Elford and Andrewes, 1936). The later claims by Bourdillon (1941), based on diffusion rate analysis, and by Chambers and Henle (1943) and Chambers, Henle, Lauffer and Anderson (1943) based on sedimentation studies, that the diameter of the influenza virus particle was only 6-11 m\(\mu\) were proved to be incorrect (Friedewald and Pickels, 1943; Stanley, 1944). Taylor et al. (1943, 1944) and Sharp et al. (1944a,b, 1945), in an extensive series, concerned themselves both with the sedimentation behaviour and appearance in electron micrographs of well studied strains of influenza virus. They obtained rather diffuse sedimentation boundaries which reflected a fairly wide distribution of particle sizes found in electron micrographs. The particle diameters as determined from the sedimentation studies ranged from 80-100 m\(\mu\). Actual measurements from electron micrographs gave
rather higher values. The values obtained for the strains PR8, LEE and Swine were 101, 123 and 97.6 m\(\mu\) respectively. Electron micrographs were obtained by Lauffer and Stanley (1944) for the strain PR8 which revealed a distribution of sizes very similar to that obtained by Sharp et al. (1944b), for which the average particle diameter was about 112 m\(\mu\). Friedewald and Pickels (1944) obtained similar values for sedimentation constant and particle size for the strains PR8 and LEE to those obtained by these previous workers, and they used their data to calculate particle weight and particle numbers present in their material. They calculated that about ten virus particles were needed to produce infection of chick embryos or mice, and at their haemagglutination endpoint the ratio of chicken red cells to virus particles was about one. In the analytical centrifuge, the sedimentation of PR8 was found to be heterogeneous for a small peak representing a lighter, more slowly sedimenting element appeared in their Schlieren diagrams. Alternatively, if this same strain was subjected to centrifugation in an artificial density gradient of sucrose, and once the sedimenting boundary had been established, was sampled for the distribution of biological activity in the tube, the material responsible for infectivity was found to be identical with the haemagglutinin and complement-fixing antigen, but a certain amount of the total haemagglutinin appeared to be non-infectious and rather lighter than the infectious material. This heterogeneity
was also encountered by Lauffer and Stanley (1944). For one sub-strain of PR8, Gard et al. (1952) found that the lighter component constituted between ten and twenty percent of the total virus. Lauffer and Miller (1944) reported that the bulk of the agglutinating activity and infectivity sedimentated at the same rate as the 100 μ component, which was also found to be homogeneous in the Tiselius apparatus.

Consequently, these studies based primarily on sedimentation behaviour in the ultracentrifuge indicated that virus activity is associated with a unit of diameter about 100 μ. This population of particles is however by no means homogeneous. In determining particle diameter in this way, knowledge of the density of the soluated particle is important. In sucrose solutions, the density of the virus particle has been found to be about 1.2 gm/ml (Elford and Andrewes, 1936; Lauffer and Taylor, 1953), a value which appears to represent the density of virus material plus water of hydration, and nothing else (Stanley and Lauffer, 1952). Sharp et al. (1944c, 1945), using bovine serum albumen solutions, obtained a density value for the virus of only 1.1 gm/ml. However, this is not a suitable type of solution in which to determine density (Lauffer and Bendit, 1954), and so the higher value appears to be correct.

With the advent of the metal shadow casting technique in electron microscopy (Williams and Wyckoff, 1945), attention shifted
to the physical appearance of the virus. Well studied strains such as PR8 and LEE were found to consist mainly of irregularly round objects, approximately 75 m\(\mu\) in diameter (Williams and Wyckoff, 1945). Occasionally elongated forms appeared which were quite distinct in size and appearance (Mosley and Wyckoff, 1946; Heinmets, 1948). These observations were confirmed by Chu, Dawson and Elford (1949), who found that these filaments were extremely rare in old laboratory strains such as PR8 and LEE. However, in recently isolated strains of virus, filaments were abundant, and rods of intermediate length were also present. The number of these elongated forms was often comparable to the number of spherical forms. In a later study Dawson and Elford (1949) measured the size and variation of the spherical particles of the strains PR8 and LEE. There was considerable variation in particle size, the mean values for diameter obtained being 90 ± 11.5 m\(\mu\) for PR8, and 103 ± 8 m\(\mu\) for LEE, which are in reasonable agreement with early values obtained by Sharp et al. (1944b), and Lauffer and Stanley (1944). Hence, direct measurements of virus diameter obtained from electron micrographs agree with values obtained by sedimentation analysis. However, even apart from the existence of filamentous forms, the virus particles are not of a uniform size. This morphological variation could reflect a variation in biological activity.

The physical nature of "incomplete virus" (von Magnus,
1947, 1951b) has also been intensively investigated. Gard and von Magnus (1947) and Gard et al. (1952) found that as the state of incompleteness increased, virus sedimented in the analytical centrifuge more slowly, and compared to the behaviour of standard virus, this sedimentation was very heterogeneous. Similar results were obtained also by Hanig and Bernkopf (1950); Pye, Holden and Donald (1956) and Paucker, Birch-Andersen and von Magnus (1959). Electron microscopic examination of incomplete virus (Werner and Schlesinger, 1954; Voss and Wengel, 1955; Pye et al., 1956; Hollós, 1957), mostly after adsorption to lysed red cells revealed flattened, pleomorphic structures somewhat larger in diameter than the infectious unit, and of lower electron density. Birch-Andersen and Paucker (1959) confirmed these findings, and also found large, bag-like structures. In ultra-thin sections of incomplete virus there was an absence of electron-dense material found in standard virus preparations.

Once the identity of the virus particle was established, it became possible to count the total number of particles present in virus preparations. Sharp (1949) and Sharp and Beard (1952) developed methods of obtaining particle counts of influenza virus based on the sedimentation of particles onto collodion or agar in the ultracentrifuge. These methods offered a means of obtaining counts for dilute preparations of virus, and the counts obtained agreed well with counts made by the sprayed droplet method of Backus and
Williams (1950). No attempt was made in either of these studies to relate particle counts to biological activity. However, particle counts were carried out by Donald and Isaacs (1954a) with the four strains PR8, MEL, A/England/1/51 and LEE, in which the total number of visible particles responsible for haemagglutination and for infectivity were determined. The particle counts were obtained both by the spray method of Backus and Williams (1950), as used by Luria, Williams and Backus (1951), and by a method of adsorbing particles onto red cell ghosts (Dawson and Elford, 1949). Regardless of strain, these authors consistently obtained ratios of the order of ten visible particles per ID<sub>50</sub>. For incomplete virus preparations of PR8, an infectious dose was represented by about 1000 particles. At the haemagglutination endpoint, they found that there was about one visible virus particle present per red cell. However, at this endpoint something less than twenty percent of the red cells are agglutinated (Fazekas de St. Groth and Cairns, 1952). So there appears to be a fivefold excess of particles present to those required to produce agglutination, if it is assumed that each particle is potentially capable of joining two red cells. In strains containing large numbers of filaments, such as A/Persian Gulf/2/52, the efficiency of haemagglutination by visible particle is about fourfold higher than for non-filamentous strains (Donald and Isaacs, 1954b), suggesting either that filaments are more efficient agglutinators than spheres,
or that some particles including filaments are capable of producing haemagglutination while others are not, although they are all capable of adsorbing to red cells. The correspondence between the number of particles required to produce infection and the number required to join red cells may be coincidental, for the active agent in each case may be represented by different classes of particles.

Attempts have been made to fractionate virus preparations into samples of different biological activity either by red cell adsorption-elution (Cohen and Smith, 1957) or by adsorption and elution from anion-exchange resin columns (Matheka and Armbruster, 1958). Although fractions differing in enzymatic activity were obtained, no other difference between these fractions was apparent.

The technique of equilibrium sedimentation in density gradients (Meselson, Stahl and Vinograd, 1957) used in this study to determine whether virus can be fractionated on the basis of density, is a means of obtaining the equilibrium distribution of macromolecular material in a density gradient which is itself at equilibrium. The density gradient is established by the sedimentation of a low-molecular-weight solute in a solution subjected to a strong centrifugal field. During centrifugation, an equilibrium is approached in which the opposing forces of sedimentation and diffusion produce a stable concentration gradient of the low-molecular-weight solute, with continuously increasing density along
the direction of the centrifugal force. Macromolecules present in such a gradient are driven to the region where the sum of the forces acting on them is zero, i.e. they seek out the region corresponding to their own density.

A major disadvantage encountered in this study was the fact that virus infectivity was inactivated by the only suitable low-molecular-weight solutes available, and consequently it was not possible to determine the distribution of virus infectivity. It was possible however to obtain distributions for haemagglutinin, enzyme, and visible particles in this way. The well studied influenza A strains MEL (Burnet, 1935) and PR8 (Francis, 1934) were used, and both standard and incomplete preparations were examined.
1. Preliminary Experiments and Technical Procedures.

Cesium chloride was chosen by Meselson, Stahl and Vinograd (1957) as the low-molecular-weight solute to use in density gradient centrifugation because being a dense material of extremely high solubility (161.4 gm/100 gm water at 0°C), solutions of high density could be obtained. The density of influenza virus has been determined to be about 1.2 gm/ml (Laufer and Taylor, 1953), and few inorganic salts are sufficiently soluble to achieve densities of this order. As the necessary density could be easily obtained with cesium chloride, and as influenza virus haemagglutinin appeared to be quite stable at these high salt concentrations, preliminary experiments were undertaken to determine the practicability of the technique for investigating the physical nature of influenza virus.

A stock solution of cesium chloride (CsCl) was prepared of density 1.642 gm/ml (5.94M). Suitable dilutions of this material with distilled water and a small amount of allantoic fluid containing influenza virus were made to give three 5 ml volumes of densities 1.17, 1.23 and 1.27. Centrifugation was then carried out in a Spinco model L ultracentrifuge using the swinging bucket rotor SW39L. In these early experiments, centrifugation was carried out for a 24 hour period at 33,000 rpm (100,000g), but in the experiments to be described later, this period was extended to 40 hours to allow complete equilibrium. At the end of this period, the con-
tents of each tube were divided into five approximately equal parts and the density and virus content of each fraction was determined. Figure 1 represents the alterations in density found over the lengths of the three tubes. By assaying each fraction for haemagglutinin, the bulk of the virus material was found associated with the fraction nearest in density to 1.25 gm/ml, and that no loss of haemagglutinin had occurred during the period of centrifugation. After centrifugation, the difference in density between the top and the bottom of the tube was found to be about 0.1 gm/ml. These experiments implied that influenza virus could be subjected to density gradient equilibrium sedimentation, so techniques for the preparation of virus and the handling of samples were investigated.

The preparation of virus stocks, their concentration by ultracentrifugation and dispersion by sonication are described in detail in the Appendix. The concentration of virus by means of fowl red cell eluates, adsorption and elution from formalinized sheep red cells, and precipitation by salts of divalent cations, such as calcium phosphate (Salk, 1941) were not reliable in our hands. Considerable aggregation results from centrifugal concentration, but a state of dispersion indistinguishable from that encountered in infected allantoic fluids can be obtained by sonication of the concentrated material for several minutes. Consequently, the virus preparations used in these studies were either
Figure 1. CsCl density gradients. (The pre-centrifugation densities of tubes 1, 2, 3 were 1.17, 1.23 and 1.27 gm/ml. respectively. Virus haemagglutinin was found to be associated with that fraction nearest in density to 1.25 gm/ml.).
unconcentrated infected allantoic fluid or virus concentrated by ultracentrifugation and resuspension.

For the collection of samples from the lusteroid tubes at the completion of centrifugation, a hole was pierced in the bottom of the tubes with a pin, and the contents of each tube were collected as single drops. Each could then be sampled for virus activity. As a preliminary check on whether any appreciable spread of boundaries occurred during movement of material down the tube, a boundary was carefully produced about half-way down a lusteroid tube between a 5 M NaCl solution and distilled water. Into the boundary region, 0.1 ml of broth containing about $2 \times 10^5$ T1 bacteriophage was carefully introduced. The bottom of the tube was then pierced and the contents collected as drops, each of which was assayed for its bacteriophage content. Figure 2 depicts the distribution of bacteriophage in the collected drops. This was characterized by a single, uniform, high peak of virus with little lateral spread, suggesting that movement down the tube does not disturb the distribution of a uniform species.

**Electron Microscopy.**

As the distribution of morphologically characteristic particles was one of the interests of this study, it was important to have a reliable electron microscopic technique for making particle counts. Ideally, the most suitable technique is one in which the virus preparations can be examined without any purification or
Figure 2. The distribution of bacteriophage in an artificially created density band. (The uniform distribution of bacteriophage banded between 5M NaCl and distilled water suggests that movement down the centrifuge tube does not disturb the distribution of a uniform species.)
concentration. A serious objection to the spraying method of Backus and Williams (1950), which was applied by Donald and Isaacs (1954a) to influenza virus, is that unless the particles are resuspended in a buffer of volatile salts, they are subject to the deleterious effects of increasing salt concentration during drying, and heavy salt crystallization in the dried droplet patterns is likely to obscure many particles. Salt concentration and crystallization does not occur if the virus suspension is spread and allowed to dry on an agar surface, for the agar adsorbs all the salt. This fact formed the basis of the agar-filtration technique described by Kellenberger and Arber (1957), where untreated virus preparations were mixed with latex suspensions and spread on collodion over agar. Microscopic examination of such preparations showed that although flattening by surface tension could not be avoided, very few artifacts were produced when compared to other methods, and the method was suitable for making particle counts.

In our hands, this later method proved to be a very satisfactory and convenient means of examining influenza virus preparations. A detailed account of the technical procedures used at each step in the preparation of micrographs suitable for obtaining virus counts is given in the Appendix. Briefly, the method adopted was as follows. Agar plates consisting of 20 ml volumes of 2% agar in water were prepared, and after drying, 0.5 ml volumes
of 0.32% collodion in amyl acetate were spread over the surface of these plates, the plates were drained of excess collodion, and again allowed to dry. The virus preparation to be examined was then mixed with a standardized polystyrene latex suspension, and carefully spread over the surface of one of these plates with a glass spreader. Filtration occurred rapidly, and the preparation was then fixed by inverting the plate over a small amount of osmic acid for fifteen minutes. After fixation, that portion of the collodion covered by the dried virus mixture was cut into small pieces, of about 5 sq. mm. in size, and floated off the agar onto copper electron microscope grids. These samples were then shadowed and photographed in an RCA EMU-3B electron microscope.

Plates 1 and 2 are representative micrographs of two separate spreadings of an undiluted PR8 infected allantoic fluid. The only preliminary treatment of this material was light centrifugation to remove coarse debris. Particles of the accepted size and appearance of influenza virus particles are seen to be abundant. These particles are quite distinct and generally show only a slight variation in diameter, so that there is virtually no uncertainty in distinguishing virus particles when counts are being made. In addition there are always a few large, flattened bizarre forms seen in these preparations which resemble forms found in mumps and influenza C virus (Isaacs and Donald, 1955) and in incomplete
Plate 1. The electron microscopy of influenza virus.

Representative micrograph of an undiluted PR8 infected allantoic fluid, prepared for microscopy by the agar-filtration technique of Kellenberger and Arber (1957).
Plate 2. The electron microscopy of influenza virus.

Representative micrograph of an undiluted PR8 infected allantoic fluid, prepared for microscopy by the agar-filtration technique of Kellenberger and Arber (1957).
virus preparations (Birch-Andersen and Paucker, 1959). A constant characteristic of such untreated virus preparations however is the relatively large number of particles associated as small groups or clusters. Such groups are a constant feature of allantoic fluid preparations and generally constitute between 10 and 15 percent of the population. It has been our practice when counting particles to regard clumps in which individual particles seem to be firmly bound together as single units. In keeping with the suggestion of Chu, Dawson and Elford (1949) that well adapted laboratory strains of influenza virus rarely contain filamentous forms, we have never encountered any filamentous forms in a large number of micrographs of both PR8 and MEL strains. In fact, compared to some published micrographs of PR8 (e.g. Ada and Perry, 1954) which show an assortment of irregular objects together with some rounded forms as representing the typical morphology of the virus, our preparations show a remarkable regularity of shape and size. This implies that little structural alteration has occurred during preparation for microscopy.

With this agar filtration technique, the thickness of the spread film and consequently the particle density varies considerably from place to place. However, Kellenberger and Arber (1957) have shown that while both the numbers of each type of particle and their ratios obtained from single micrographs vary considerably, if five separate counts are made from different
fields of the specimen, the precision of the method is in the same range as achieved by the spray droplet technique. Consequently, in determining particle titres for influenza virus, counts were made from at least five fields.

Table 1 represents values for particle and haemagglutinin titres of four separate PR8 allantoic fluids.

Although the values obtained are slightly lower than those obtained by Donald and Isaacs (1954a), at the agglutination endpoint there is still a considerable excess of particles to agglutinated red cells, for the total number of red cells added per tube in these tests varies between about $10^7.1$ and $10^7.2$. Similarly, infectivity tests on virus preparations such as these, carried out by the method of Fazekas de St. Groth and White (1958a), invariably show a ten to twenty-fold excess of visible particles to infectious units.

Inactivation of Infectivity by Cesium Chloride.

After it was established that influenza virus could be banded in CsCl density gradients, it was found that although virus haemagglutinin appeared to be completely stable through a wide range of ionic strength, there was a marked loss of virus infectivity on storage in concentrated CsCl solutions. That this inactivation was not due to high ionic strength alone was borne out by the fact that storage of virus in similar concentrations of sodium chloride does not result in any marked loss of infect-
TABLE 1.

THE NUMBER OF PARTICLES REQUIRED
TO PRODUCE HAEMAGGLUTINATION.

<table>
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<tr>
<th>PR8 Preparation</th>
<th>HA/ml ($\log_{10}$)(^a)</th>
<th>P/ml ($\log_{10}$)(^b)</th>
<th>P/HA(^c)</th>
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<tr>
<td>1</td>
<td>$10^{3.75}$</td>
<td>$10^{10.81}$</td>
<td>$10^{7.06}$</td>
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<td>2</td>
<td>$10^{3.36}$</td>
<td>$10^{10.54}$</td>
<td>$10^{7.18}$</td>
</tr>
<tr>
<td>3</td>
<td>$10^{3.59}$</td>
<td>$10^{10.52}$</td>
<td>$10^{6.93}$</td>
</tr>
<tr>
<td>4</td>
<td>$10^{2.68}$</td>
<td>$10^{9.69}$</td>
<td>$10^{7.01}$</td>
</tr>
</tbody>
</table>

\(a\) = Haemagglutinin titre  
\(b\) = Particle titre  
\(c\) = Number of particles representing one agglutinating unit
ivity. Infectivity also remains high if virus is kept in 
physiologically "normal" (0.147 M) CsCl solutions. However, 
all attempts to associate this inactivation with a trace im-
purity, or to counteract empirically this effect failed. Loss 
of infectivity could not be prevented by the addition of chelating 
agents, proteins such as gelatine or albumen, or adsorbents such 
as charcoal or starch to 2 M CsCl solutions containing virus. 
Alteration of hydrogen ion concentration between pH 6.0 and 8.0 
under these conditions similarly had no effect. However, the 
presence of high concentrations of some related cations, e.g. 
3.9 M potassium chloride or 2.1 M lithium chloride resulted in 
a smaller loss of infectivity. The loss was only 80 to 200 fold 
in the presence of these salts and 3.0 M CsCl, whereas it was grea-
ter than 10,000 fold in their absence.

That this inactivating effect was due to the cesium ion 
was shown by the partition of CsCl on a cation-exchange resin 
column. Cesium chloride was added to an acidified Dowex-50 resin 
column, where cesium ions replaced hydrogen ions, and the chloride 
ions of the salt passed through the column in the form of hydrogen 
chloride. This was converted to sodium chloride by neutralization 
with sodium hydroxide, and evaporated to dryness. This fraction 
contained the original chloride ions of the cesium salt. Cesium 
was then removed from the column by addition of 50% hydrochloric 
acid, in which case the hydrogen ions replace the cesium ions on
the column, and cesium chloride is released. This fraction was also dried. The two dried fractions, one containing the original cesium ions and the other the original chloride ions were then made up in solutions of equivalent strength to that of stock CsCl solution, and tested for their capacity to inactivate virus. The effects of these fractions in inactivating infectivity is shown in Table 2 where X represents in each case the ions obtained by partition. The mixtures shown in the Table were kept overnight in the refrigerator before testing for infectivity. The virus was in the form of PR8 infected allantoic fluid.

The amounts shown in each column represent the volume in ml of each constituent. Clearly, extensive inactivation is occurring only with those samples which contain a high concentration of cesium ions.

The mechanism of virus inactivation by cesium is not subject to simple explanation, as is seen from Figure 3, which depicts the rate of virus inactivation on storage in 2M CsCl.

As it has been impossible to prevent this inactivation of infectivity by cesium chloride, or to find an adequate substitute for cesium, no information could be obtained on the distribution of infectivity in virus stocks. The bromide salts of the alkali metals were considered as possible substitutes for cesium chloride as they are capable of producing solutions of high density, but they likewise inactivated virus infectivity. The
TABLE 2.

THE INACTIVATION OF VIRUS INFECTIVITY BY CsCl.

Identification of the agent responsible for inactivation.

| Sample | Constituents | Infec-
|        |              | tivity
|        | Virus | Distilled | CsCl 6M | NaCl 6M | XCl 6M | NaX 6M | Titre log10 |
|        | 0.1 | 0.2 | - | - | - | - | 7.2 |
| CsCl   | 0.1 | 0.1 | 0.1 | - | - | - | 4.6 |
| NaCl   | 0.1 | 0.1 | - | 0.1 | - | - | 6.8 |
| XCl    | 0.1 | 0.1 | - | - | 0.1 | - | 4.9 |
| NaX    | 0.1 | 0.1 | - | - | - | 0.1 | 7.0 |

The partition of CsCl on a cation-exchange resin column.

The letter X represents the ions obtained from this partition.
Figure 3. The inactivation of influenza virus infectivity by cesium chloride.
salts of divalent metals were not considered as they generally produce precipitation of the virus. There were no detectable virus strain differences to the inactivating effect of CsCl.

The inactivation of influenza virus by cesium chloride does not seem to be the result of osmotic shock. There was no difference in the degree of inactivation produced when 3 M solutions of cesium chloride containing virus were slowly or very rapidly diluted into distilled water.

2. The Distribution of Haemagglutinin.

To obtain distribution of virus near the centre of the tubes, an initial density of the virus - cesium chloride mixture of 1.24 was aimed at. After centrifugation for 40 hours at 33,000 rpm, drop samples were collected into one ml volumes of distilled water. Tests for haemagglutinin were carried out in the manner described by Fazekas de St. Groth and Graham (1955), except that fowl red cells were added in the form of 0.25 ml volumes of a 1% suspension, with an automatic pipette. The error of a single titration carried out in this way is very small, being $\pm 0.11 \log_2$ units, or about 8%. For graphical comparison, all results are plotted as equal area curves, i.e. the distributions of haemagglutinin in all cases are represented by the same area, and are directly comparable.

**Standard Virus.**

Fully infectious virus preparations, designated as
standard virus (von Magnus, 1951a) were prepared in the conventional manner, and subjected to density gradient centrifugation. Representative distributions of haemagglutinin for the influenza A strains MEL and PR8 at equilibrium are shown in Figures 4 and 5.

Meselson, Stahl and Vinograd (1957) have shown that the concentration distribution at equilibrium of a single macromolecular species is Gaussian, whereas the distribution becomes skewed in the presence of material heterogeneous with respect to effective density. Standard MEL virus is characterized by a slightly skewed, unimodal band, but more than 99% of the total haemagglutinin is concentrated in a narrow band, while a small fraction of the material displays a heterogeneous array of lower densities. With standard PR8 virus, the distribution is considerably more skewed, with approximately 20% of the virus material constituting the tail region. Again, the haemagglutinating activity is in a narrow band. It is possible however that this distribution may be bimodal, characterized by a high peak representing a relatively homogeneous virus fraction overlapped by a diffuse distribution of particles heterogeneous in respect to density, with the differences in effective density being insufficient to result in the formation of discrete bands. On the other hand, despite the fact that preliminary experiments with bacteriophage suggested that very little mixing occurs on sampling, it was considered possible that at least part of the skewness of these distributions may have been
Figure 4. The equilibrium distribution of haemagglutinin for a standard MEL preparation in a cesium chloride density gradient. The initial density of the virus - cesium chloride mixture was 1.24 gm/ml.
Figure 5. The equilibrium distribution of haemagglutinin for a standard PR8 preparation in a cesium chloride density gradient. The initial density of the virus - cesium chloride mixture was 1.24 gm/ml. This curve is of equal area to that shown in Figure 4.
the result of diffusion or carry over during sampling. Consequently, sample No. 35 from the homogeneous peak of the PR8 run was reband and examined for haemagglutinin distribution. Figure 6 shows the distribution obtained. Clearly, this curve indicates that an almost normal distribution can be expected from the banding of a homogeneous species, and that very little carry over occurs.

Hence, the haemagglutinin distributions for normal virus preparations show that while the bulk of the virus haemagglutinin is fairly homogeneous with respect to density, there is a variable amount present, dependent on the strain, which is less dense than the rest to a variable extent.

Incomplete Virus.

Stocks of first and second passage incomplete PR8 virus were prepared as described in the Appendix and subjected to density gradient centrifugation. The results obtained for first passage virus are shown in Figure 7. Once again a unimodal skewed distribution occurs, but the peak of haemagglutinin activity is not nearly so prominent as in standard virus preparations and is considerably wider, suggesting that the bulk of this type of virus is more heterogeneous with respect to density than is standard virus. Also present again is the diffuse class of particles of lower density found with standard virus, constituting a prominent tail. With further passage, the heterogeneity of density increases, as shown by second passage incomplete virus in Figure 8. In this
Figure 6. The re-banding of material showing density homogeneity. The equilibrium distribution obtained for fraction No. 35 of the standard PR8 preparation shown in Figure 5. The black band indicates the original area occupied by this sample.
Figure 7. The equilibrium distribution of haemagglutinin for a first passage incomplete virus preparation of PR8. This curve is of equal area to that shown in Figures 4 and 5.
Figure 8. The equilibrium distribution of haemagglutinin for a second passage incomplete virus preparation of PR8.
case, no uniform fraction can be distinguished and the resultant curve can be best described as representing a broad spectrum of density types, with a general shift towards lower density. A very light fraction is also present which floats on the top of the gradient. Consequently, the lowering of infectivity and loss of morphological uniformity which have been observed to occur on undiluted passage of standard virus preparations are associated with a marked increase in heterogeneity of particle density. Figure 9 is a composite of standard, first and second passage incomplete virus plotted to demonstrate these alterations in density heterogeneity on passage. In the originals, the positions of each band in the tubes varied slightly, due no doubt to small, unavoidable differences in the initial densities of the mixtures. In this figure, they have been adjusted by assuming that the first detectable appearance of haemagglutinin occurs at similar densities.

3. The Distribution of Enzyme.

The haemagglutinating activity of influenza virus is due to a binding between the virus borne enzyme, neuraminidase (Gottschalk, 1958), and mucoprotein substrate molecules present on the surface of red cells. Ether treatment of influenza virus (Hoyle, 1952) breaks down the virus particles into much smaller units, but considerably increases the haemagglutinin titre of such preparations, which suggests that the intact virus particle contains a considerable
Figure 9. The relative equilibrium distributions of standard virus and first and second passage incomplete virus preparations of PR8.
amount of this enzyme. In order to determine if all virus particles contain the same amount of enzyme, distribution of enzyme for several preparations of PR8 which had been subjected to density gradient centrifugation was determined, and compared to the corresponding distribution of haemagglutinin obtained for these preparations.

Tests for enzyme activity were determined using ovomucin as substrate. The ovomucin was prepared from egg white and titrated for inhibitory activity as described by Gottschalk and Lind (1949). The test for enzyme activity followed that described by Stone (1949), in which five inhibitory units of ovomucin were incubated with serial two-fold dilutions of virus at 37°C for one hour, followed by 30 minutes heating at 65°C to destroy further virus activity. Residual inhibitory activity was then determined by addition of five agglutinating doses of heat-inactivated LEE virus, followed 30 minutes later by 0.25 ml of a 1% suspension of "sensitive" fowl red cells (Anderson, 1948). These methods are described in detail in the Appendix.

Distributions obtained for both enzyme and haemagglutinin for two different preparations of PR8 are shown in Figures 10 and 11. The actual values obtained in haemagglutinin units or enzyme units have been corrected to values which when plotted give curves of equal area to those shown in Figures 4 and 5. The curves drawn in Figures 10 and 11 (open circles) represent the distributions of
Figure 10. The equilibrium distribution of virus enzyme and haemagglutinin for a standard PR8 preparation. Closed circles represent the values obtained for enzyme; open circles indicate the values obtained for haemagglutinin.
Figure 11. The equilibrium distribution of virus enzyme and haemagglutinin for a standard PR8 preparation. Closed circles represent the values obtained for enzyme; open circles indicate the values obtained for haemagglutinin.
haemagglutinin, while the closed circles represent the corresponding enzyme values. The distributions of haemagglutinin for these standard PR8 virus preparations closely resemble that shown in Figure 5. The enzyme distribution obtained in both cases shows a similarity to that obtained for haemagglutinin, although there is often a considerable discrepancy between individual values. The general tendency of the enzyme distribution of Figure 11 to be shifted slightly to the left of the haemagglutinin distribution is not found in Figure 10. The variability of individual values is probably a reflection of the inherent variability of the test for enzyme, and it is likely, for example, that the value obtained for sample 30 in Figure 10 represents a titration error, rather than a significant difference and hence the correspondence between enzyme and haemagglutinin may be closer than shown. There appears to be as much enzyme associated relatively with virus of lower density as there is with the virus of higher density.

4. The Distribution of Visible Particles.

As a suitable electron microscopic technique for particle counts was developed late in the course of these studies, the only distribution of particles examined was obtained from the fractions of a standard MEL preparation after density gradient centrifugation (Figure 4). This was an unfortunate choice in searching for possible particle heterogeneity, as the haemagg-
lutinin distribution for strain MEL is much more homogeneous than for PR8, and morphologically the virus contains much fewer of the large, flattened bizarre forms seen quite commonly in PR8 preparations. Particle counts were undertaken on samples 19, 21, 23, 25, 27, 29, 31 and 47 of the MEL distribution shown in Figure 4. Other samples were taken from the tail region, but too few particles were present for counts to be made. Table 3 lists the number of visible particles per ml found for this strain before centrifugation and for the various fractions examined, as well as the ratios of particles to haemagglutinin. Columns showing the actual number of virus and latex particles counted are included in this table.

Again for this strain, more particles are found to be present at the haemagglutination endpoint than there are joined-red cells. The ratio of particles to haemagglutinin varies over about a twofold range throughout the distribution with the exception of sample 47 and although when the particle distribution is plotted against the haemagglutinin distribution it appears to be shifted slightly to the right (Figure 12), the correspondence of the two is close. However, at the extreme right hand end of the distribution, the extent of the excess of virus particles rises from about fourfold to about twentyfold. Micrographs of virus located in this portion of the band show that there is much greater variation in the size of particles than in the peak
The relation of the distribution of total particles to the distribution of haemagglutinin after density gradient centrifugation.

<table>
<thead>
<tr>
<th>MEL preparation</th>
<th>V^\text{a}</th>
<th>L^\text{b}</th>
<th>P/ml(\log_{10})^\text{c}</th>
<th>HA/ml(\log_{10})^\text{d}</th>
<th>P/HA(\log_{10})^\text{e}</th>
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<tr>
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<td>10.48</td>
<td>3.81</td>
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<tr>
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<td>203</td>
<td>11.26</td>
<td>4.31</td>
<td>6.95</td>
</tr>
<tr>
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<td>240</td>
<td>11.04</td>
<td>4.01</td>
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<tr>
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<tr>
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<td>404</td>
<td>10.18</td>
<td>2.68</td>
<td>7.50</td>
</tr>
</tbody>
</table>

\text{a} = Virus particle count obtained from five fields.
\text{b} = Latex particle count obtained from five fields.
\text{c} = Virus particle count per ml.
\text{d} = Haemagglutinin titre.
\text{e} = Number of particles representing one agglutinating unit.
\text{f} = Virus preparation diluted tenfold before mixing with latex.
Figure 12. The relative distributions of total virus particles and virus haemagglutinin for a standard MEL preparation. The open circles represent the distribution of visible particles, while the closed circles represent the distribution of haemagglutinin.
samples. The presence of large numbers of particles rather smaller than the normal virus particle was responsible for the high particle to haemagglutinin ratio. It is quite likely that many of these particles do not represent virus, but particulate matter of lower density which floats at the top of the density gradient. Consequently in plotting the relative distribution of particles to haemagglutinin shown in Figure 12, this value of sample No. 47 was not considered. (In this figure, the closed circles represent the percentage of the total haemagglutinin found in each fraction, while the open circles represent the percentage of the total particles obtained for corresponding fractions.) The overall impression gained from particle counts is that the distribution of visible particles is identical to the distribution of biological activity, so that there is no evidence for the existence of a class of biologically inert particles, which have a different density distribution from the biologically active particles.
DISCUSSION.

The aim of this study was to see whether the particle count discrepancy encountered by Donald and Isaacs (1954a) was wholly or in part due to the existence of different types of particles. The problem is of considerable importance for biological studies of the multiplication of the virus where it is necessary to have a true estimate of how many particles are actually responsible for a given effect. The technique of density gradient centrifugation seemed a likely means of investigating the problem.

Influenza virus has a density of about 1.25 gm/ml in cesium chloride solutions, a value which is higher than that of 1.19 gm/ml found in sucrose solutions (Lauffer and Taylor, 1953). This is probably due to the penetration of the mantle of hydration of the virus particle by cesium ions.

Although both tobacco mosaic virus and bacteriophage have been banded by this method without loss of infectivity (Meselson, Stahl and Vinograd, 1957), and concentrated solutions of cesium chloride do not affect the infectivity of poliovirus or vaccinia, it has been impossible to prevent the inactivation of influenza virus. The reason for this inactivation is not clear. During this study, attempts were made to substitute saturated sodium chloride - deuterium oxide solutions, with which an initial density of 1.25 gm/ml can just be reached, for cesium chloride
solutions in the hope of preventing this loss of infectivity. However, after centrifugation in this mixture, not only was there again a considerable loss of infectivity, but the density gradient produced was so slight that virus was always distributed right throughout the tube, although there was greater concentration near the bottom than at the top. Consequently the infectivity distribution in banded material could not be determined.

The distribution of visible particles suggests that there is no evidence for the existence of totally inactive virus, unable to be detected by either infectivity or haemagglutination. For the strain MEL, even though it shows little density heterogeneity, the distribution of visible particles is very close to the distribution of haemagglutinin and enzyme. So with some inefficiency, haemagglutinin is a measure of the total virus particle concentration. Consequently, for strain MEL, though infectivity cannot be measured, infectivity must reside in the main peak, since there are not enough virus particles outside that peak to constitute the infective fraction. By analogy, infectivity is probably likewise associated with the major peak of haemagglutinin activity for strain PR8. The twenty percent fractions of PR8 haemagglutinin which is of lower density than the bulk is probably identical to the lighter fraction encountered in sedimentation studies (Friedewald and Pickels, 1944; Lauffer and Stanley, 1944; Hanig and Bernkopf, 1950; Gard et al., 1952).
It is possible that infectivity could be associated with either the bulk of the virus which is of greatest density, or with the lower density material. However, Friedewald and Pickels (1944) found that their lighter fraction was non-infectious haemagglutinin, and so the less dense material found on density gradient centrifugation also probably represents non-infectious material. The inference that can be drawn therefore is that while standard virus preparations of influenza virus are not entirely homogeneous with respect to density, all particles are probably able to haemagglutinate and the bulk of them are presumably potentially infectious. Consequently, the particle count deficiency may be attributable to inherent shortcomings in the method of assay. It is possible on the other hand that there is no correlation between particle density and capacity to haemagglutinate. This separation method may have simply failed to separate active from inactive particles.

The relatively homogeneous nature of standard virus preparations is not found for incomplete virus preparations. As the degree of incompleteness increases, heterogeneity becomes very marked. Once again, there is a close parallel to the findings obtained by direct sedimentation. Gard et al. (1952) found that the faster moving, homogeneous material present in the strain PR8 fell to about 50% of the total in first passage incomplete material, and the amount of slow moving material increased pro-
portionately, though the latter was not at all homogeneous.
Similarly, second passage material in the analytical centrifuge contained only about 5% of the fast component, and a diffuse distribution of lighter material. The distribution of first and second passage incomplete virus in density gradients could be interpreted the same way. It is impossible, however, in the case of incomplete virus preparations to draw any inferences as to the location of the residual infectivity. It seems likely however that infectivity is associated with the fraction of greatest density.
SUMMARY.

Preparations of standard influenza A virus were subjected to equilibrium sedimentation in density gradients of cesium chloride solutions as a means of investigating the significance of the surplus visible particles found in electron microscope counts of such preparations. The physical nature of incomplete virus preparations was also examined by the same technique.

The distribution of visible particles obtained for one standard virus preparation was similar to the haemagglutinin distribution. So, haemagglutination, with some inefficiency reflects the distribution of total particles. Consequently, the particle discrepancy may reflect deficiencies in the assay methods (there being no predetermined difference between particles), or on the other hand there may be no correlation between density and capacity to haemagglutinate (the method simply failing to distinguish between active and inactive). However, at least there is no evidence to suggest that a separate class of inactive particles exists.

Similarly, the distribution of virus enzyme suggests either that it is uniformly distributed in the virus population, or that there is no correlation between virus density and enzyme distribution.

Standard virus preparations consist mainly of particles
of similar density, although a small class, whose magnitude varies according to strain, of particles of differing density is always present.

Incomplete virus preparations lack the homogeneity of standard virus preparations. As incompleteness increases, an array of particle types of variably lower density appears.

Infectivity distributions could not be determined but this property must be associated with the predominant density type present in standard virus preparations, and which appears to be progressively lost in incomplete virus preparations.
SECTION II

THE BIOLOGY OF INFLUENZA VIRUS
GENERAL INTRODUCTION.

Influenza virus is capable of multiplying in the allantoic cells of the developing chick embryo (Nigg, Crowley and Wilson, 1940; Burnet, 1940), with subsequent release into the allantoic fluid (Nigg, Crowley and Wilson, 1941; Burnet, 1941; Henle and Chambers, 1941). This provides a suitable laboratory system in which to study the fundamental aspects of influenza virus multiplication, and a means of titrating virus infectivity. The virus is also able to agglutinate chick red cells (Hirst, 1941; McClelland and Hare, 1941). Haemagglutination is a simple means of detecting virus, and provides another quite independent assay method for determining virus activity. Use of these techniques has resulted in the accumulation of a great deal of information about the mechanics of virus multiplication. Without attempting to review the whole subject, a few significant features of this process might be mentioned before discussing the biological aspects of virus multiplication with which this thesis is concerned.

The uptake of influenza virus into the cells of the allantois takes place in two stages. Within seconds of inoculation, the virus becomes attached to the surface of the cells (Henle and Henle, 1944b; Henle, Henle and Kirber, 1947). This rapid attachment to receptors is followed by the gradual uptake of virus extending over several hours (Fazekas de St. Groth,
1948). The primary attachment of virus to receptors is an enzyme-substrate union, and adsorption can be entirely prevented by pretreatment of the cells with the receptor destroying enzyme - RDE (Stone, 1948). The second stage of penetration into the cell is not dependent on the integrity of the enzyme, for enzyme activity can be destroyed by heating, or the receptors can be modified with periodate to a state in which they are insusceptible to enzyme action, and virus can still gain entry to the cell. This second stage has been called viropexis (Fazekas de St. Groth, 1948). Once inside the cell, the virus particle disintegrates (Hoyle and Frisch-Niggemeyer, 1955) and during much of the period of intracellular multiplication, no virus components can be detected (Hoyle, 1948; Henle, 1949). Maturation of virus and its release from cells is almost instantaneous, if cell receptors have been removed with RDE. There is a delay in release, however, associated with the time of elution, when cell receptors are present (Cairns and Mason, 1953). The first appearance of virus progeny is dependent on the amount of virus initiating infection. There is a highly variable delay at the beginning of multiplication in individual cells which seems to be characteristic for each virus-cell encounter (Cairns, 1957). An increase in the number of particles infecting each cell greatly increases the synchrony of virus release, and so hastens the appearance of virus. Under conditions where starting delay
is minimized, the period of intracellular multiplication occupies about three to four hours (Cairns, 1957), and release of virus from individual cells is extended over several hours.

There are three major aspects of the biology of influenza virus with which we shall be concerned, and they are all associated with multiple infection of cells by virus.

The first problem concerns the conditions of production and the significance of "incomplete" virus formation. As mentioned elsewhere, the inoculation of large quantities of influenza virus into the allantoic cavity results in the production of non-infectious haemagglutinin (von Magnus, 1947; 1951b). This virus, although incapable of further multiplication or perhaps capable of only a few cycles of multiplication (Burnet, Lind and Stevens, 1955), nevertheless shows the normal features of haemagglutination and antigenicity (von Magnus, 1951c). The physical characteristics of this type of virus have been outlined in the first section of this thesis.

The second problem bears some relation to the nature of genetic interactions between influenza viruses. The demonstration of genetic recombination between related bacterial viruses (Delbruck and Bailey, 1946) provided an incentive to seek similar interactions between animal viruses. As influenza A strains possessed a number of easily tested "marker" characteristics by which individual strains could be differentiated, evidence for
recombination was sought for among these different strains (Burnet and Lind, 1949), and the problem has subsequently been dealt with by conventional genetic methods and terminology. This subject has recently been extensively reviewed (Burnet, 1958; 1959). While it has been possible to demonstrate that the progeny of mixed infections can contain characteristics of each parental type and this has been called genetic recombination, closer examination reveals a complex situation. Other effects, as well as what appears to be true genetic recombination, occur. These include heterozygosis, phenotypic mixing and the appearance of unstable forms which cannot be maintained as clones (Burnet, 1959). Any analysis of the distribution of virulence among progeny particles is complicated by the fact that most of this progeny may in fact be incomplete virus. Technically, it is impossible to determine whether the "marker" characteristics used in such studies truly represent the phenotypic expression of single, distinct hereditary units, or much larger numbers of loci. For reasons such as this, a method of examining what happens when two virus particles enter the same cell and collaborate in the production of progeny which is not dependent on the analysis of "marker" interchange is desirable. Multiplicity reactivation or the interaction of two or more inactivated particles to produce progeny where individually such particles are incapable of doing so, provides such a method. The demonstration
of multiplicity reactivation for influenza virus, and a study
of the manner in which it occurs is the approach adopted to
this problem in this thesis.

Finally, the third problem is associated with the
nature of viral interference. Interference has been defined
as the antagonistic or inhibitory effect produced by one virus
upon the propagation of another (Henle, 1950). In the partic­
ular case of influenza virus, it was found that both active and
inactive virus could prevent or diminish the multiplication of
subsequently inoculated active virus (Andrewes, 1942; Henle
and Henle, 1943; 1944a; Zeigler and Horsfall, 1944; Zeigler
et al., 1944). This blocking effect is most easily studied
using inactivated virus as interfering agent. It has been estab­
lished that such interference occurs intracellularly and is not
merely the result of the destruction of surface receptors (Isaacs
and Edney, 1950b). Despite the fact that the interfering agent
is rapidly adsorbed (Henle and Henle, 1944b), interference is
not fully established until about twelve hours later (Fazekas
de St. Groth, et al., 1952). With extremely large doses, this
time interval may be reduced to nine hours, while conversely
small interfering doses require at least twenty-four hours to
become established (Henle and Paucker, 1958). Once established,
cells remain in the interfered state for at least 96 hours
(Henle and Henle, 1944b). Inactivation of virus by heat or
formalinization is apt to destroy most of the interfering capacity together with the infectivity (Henle, 1950). Virus inactivated by ultraviolet irradiation is the most commonly used source of interfering agent. An investigation of the nature of heterologous interference, i.e. interference between different types of influenza virus, constitutes the last section of this thesis.

A serious drawback to the quantitative study of such problems as these has been the lack of a reliable estimate of the number of allantoic cells. Early estimates obtained for chick embryos eleven to twelve days old, either by direct cell counts or biologically (Hoyle, 1948; Henle, 1950; Fazekas de St. Groth and Cairns, 1952) proved to be too high (Tyrrell et al., 1954). However, Cairns and Fazekas de St. Groth (1957) have recently provided accurate estimates of cell numbers for embryos between eight and fifteen days of age, based on counts of fixed and stained preparations of large numbers of embryos, which greatly reduces this uncertainty.

The intact chick embryo is a rather cumbersome experimental system. A useful simplification in the form of the de-embryonated egg was introduced by Bernkopf (1949). The contents of the egg are tipped out and the virus is cultivated in that part of the allantoic membrane which remains adherent to the shell. The de-embryonated egg is extensively used in recom-
bination experiments (Burnet, 1958).

Attempts have been made to use small pieces of chorio-allantoic membrane removed from eggs and suspended in a suitable medium as host material for titrations of virus infectivity (Fulton and Armitage, 1951). This method was, however, 20 to 80 times less sensitive than titration in whole eggs. By cutting the shell of de-embryonated eggs into small pieces with adherent chorio-allantois, Fazekas de St. Groth and White (1958a, c) have succeeded in producing an infectivity method which is equal in sensitivity to whole eggs. Each small piece is the equivalent of one whole egg in such a titration. Not only can more replicates be used per dilution step, with a corresponding increase in titration accuracy, but the response obtained is Poissonian (Fazekas de St. Groth and White, 1958b). The use of bits in this way virtually eliminates the problem of egg to egg variation in susceptibility encountered in the use of whole eggs, and felt to be the cause of the flattening of the dose-response curve (Fazekas de St. Groth and Cairns, 1952).

The successful substitution of surviving pieces of allantois-on-shell for whole eggs in infectivity titrations suggested that they may provide a useful experimental system in which to examine the problems mentioned above. Most of the following studies have been carried out in such a system, which employs standard sized pieces of allantois, and which has proved
to be reproducible and easy to handle. The multiplication of influenza virus in this system will be considered in relation to the problem of incomplete virus formation. A major advantage of this system has proved to be that large numbers of susceptible cells can be obtained for experimental use with very little effort.
CHAPTER I.

THE PRODUCTION OF INCOMPLETE VIRUS.

INTRODUCTION.

The concept of incompleteness was introduced by von Magnus (1947; 1951b) to account for the non-infectious but haemagglutinating virus which appears if large inocula are used for virus production. Whenever high dilutions of infected allantoic fluid are used for preparing virus, a relatively constant relation between the infectivity and haemagglutinin titres of the yields is obtained. This $\frac{ID_{50}}{HA}$ ratio usually has a value greater than $10^6$ (Henle and Henle, 1949; Hoyle, 1950; von Magnus, 1951a; Fazekas de St. Groth and Cairns, 1952), and such preparations are conventionally regarded as being fully infectious. On the other hand, when undiluted allantoic fluid is used as inoculum, the yields show a reduction in this $\frac{ID_{50}}{HA}$ ratio (von Magnus, 1947; 1951a). Furthermore, if such yields are passaged as undiluted allantoic fluid, there is a progressive reduction in the $ID_{50}/HA$ ratios of successive yields. The haemagglutinin titres of these preparations remains fairly constant, and it is a loss of infectivity which results in low ratios. von Magnus (1951b; 1952) suggested that this non-infectious, haemagglutinating material may be intermediate,
immature form of fully active virus, and coined the term "incomplete" to describe it. Despite the restriction in meaning imposed by this definition, the term "incomplete" has come into general use as a descriptive term for such preparations. Henle and Henle (1949) had encountered this phenomenon in studies designed to show that influenza virus developed through an immature stage to fully infectious virus. Their notion of gradual maturation probably led von Magnus to assume that he was dealing with a precursor form. The idea of an unfinished cycle of multiplication, in which the developmental process has been interrupted at some stage, as distinct from an imperfect cycle of multiplication resulting in a faulty product, unnecessarily limits the possible significance of this phenomenon. The general idea of aberrant multiplication implicit in this von Magnus phenomenon, however, has attracted widespread interest. One implication of the idea that this virus is imperfect rather than immature is that it is uniformly avirulent. This was suggested by Fulton (1953). However, the infectivity behaviour of the small infective fraction of incomplete virus preparations is indistinguishable from that of normally active preparations (Fazekas de St. Groth, 1955), and the simplest conclusion that could be reached as to the nature of such preparations is that they consist of a mixture of infective and non-infective forms. However, during one cycle of multiplication,
incomplete preparations are capable of producing far more haemagglutinin than would be expected from their infective virus contents. Consequently, non-infective particles must be participating in the production of haemagglutinin. Burnet, Lind and Stevens (1954; 1955) suggested that the most likely interpretation is that classes of incomplete particles exist which are incapable of unlimited multiplication and are unable therefore to score as positives in infectivity titrations, but yet are capable of initiating one or several cycles of non-infectious, haemagglutinin production. The most incomplete form is then represented by particles which are incapable of undergoing any further multiplication. Besides being non-infectious, incomplete forms differ physically in many ways from active virus. As the degree of incompleteness increases, the virus sediments more slowly and diffusely in the analytical centrifuge (Gard and von Magnus, 1947; Hanig and Bernkopf, 1950; Gard et al., 1952; Pye, Holden and Donald, 1956; Paucker, Birch-Andersen and von Magnus, 1959); it is also less dense and more heterogeneous (Section I). Morphologically, it is flattened, pleomorphic and less electron dense (Werner and Schlesinger, 1954, Voss and Wengel, 1955; Pye et al., 1956; Hollos, 1957), and lacks electron dense material in cross-sections (Birch-Andersen and Paucker, 1959). The amount of RNA associated with such preparations decreases with increasing incompleteness (Ada and
Perry, 1956), and on ether disruption such particles possess less haemagglutinin than is possessed by infectious particles (Paucker, Birch-Andersen and von Magnus, 1959). In short, under certain growth conditions in the allantois, a wide range of defective forms of influenza virus can be produced.

Attempts to define the conditions necessary for producing this phenomenon, knowledge of which is essential for interpreting what is happening, have led to considerable controversy. This has centred around the widely held belief that multiple infection of cells is of prime importance in the production of incomplete virus. von Magnus (1952; 1954) developed such an explanation around his notion of the formation of a precursor form, and felt that partial interference by incomplete forms which he assumed to be present even in complete preparations in small amounts, with active virus under conditions of multiple infection in some way prevented the conversion of newly formed but immature virus into fully active virus. It is implicit in this auto-interference theory, which has been treated theoretically by Gard (1953), that more concentrated inocula, by virtue of giving higher multiplicities of infection, should result in progressively less complete virus appearing. However, Cairns and Edney (1952) in a dose response experiment showed that once incomplete virus production was occurring a thousandfold change in the initial inoculum was not followed by any significant
variation in the amount of infectious virus produced. They also found that incomplete virus was appearing in their experiments at estimated multiplicities as low as 0.02, i.e. when only two per cent of the cells were infected, and so they rejected the multiple infection hypothesis and postulated the existence of an unknown factor available initially in only limited amounts and whose depletion with moderately large inputs resulted in incomplete virus formation. Unfortunately, their ingenious multiplicity estimates considerably overestimated the actual number of cells present in the allantois, for no allowance was made for asynchrony (Cairns, 1957). Their multiplicity estimates were set by the position of the bend over point of the dose response curve. However, in this type of experiment which is stopped before all cells have yielded, relatively more multiply infected cells than singly infected cells will have produced yield. Consequently, the bend over point of the response curve does not represent the point at which multiple infection is occurring, but some higher value. A correction for this would place the critical multiplicity much higher.

A complication to any interpretation is introduced by the variable behaviour of different influenza strains. Different strains of virus have been found to yield considerably different proportions of incomplete offspring (von Magnus,
strains can be graded in order of decreasing ability to produce incomplete virus in either the allantois or mouse lungs, and in either case the gradient order is different (Fazekas de St. Groth and Graham, 1955). There is a close resemblance between the "incomplete gradient" for the allantois, and the gradient of enzyme action of these viruses on the allantoic membrane (Fazekas de St. Groth and Graham, 1954a). Fazekas de St. Groth and Graham (1954b; 1955), influenced by the "missing substance" hypothesis of Cairns and Edney, suggested that virus enzyme substrate may play the essential role of inducing the production of some host cell constituent which is necessary for completeness. If this were absent, non-infectious virus would result. This hypothesis suggests that the ability of virus to modify receptors during adsorption may be the necessary condition for incomplete virus production. Strong evidence in support of this was provided by the finding that if virus receptors were modified with metaperiodate, not only did non-infectious virus appear at much lower multiplicities than previously, but strain differences also disappeared. These findings imply that incomplete virus formation is independent of multiplicity and that it is an inherent property of the virus dependent on its enzymatic activity.

In the meantime other workers, although hampered by
the absence of reliable multiplicity estimates, favoured the multiple infection hypothesis. Considerable attention was paid by these workers to factors of dosage and timing, and to the effect of the presence of thermally inactivated particles in inocula. However, their attention was confined to the single strain PR8. Horsfall (1954), using preparations as free from thermally inactivated virus as possible, found that inputs of $3 \times 10^7$ or more infective particles produced incomplete virus. Production of incomplete virus also occurred for similar inputs of virus partially or completely inactivated after storage at $35^\circ C$ or $22^\circ C$. These experiments were carried out in ten day old chick embryos, so that these multiplicities seem to be something less than one per cell. Henle (1953) had previously reported the ability of heat inactivated virus to participate in incomplete virus production. Similar results to those of Horsfall were obtained by Pinter, Liu and Henle (1955), who, however, obtained a value of $10^6$ ID$_{50}$ as the critical multiplicity. This would constitute a multiplicity considerably greater than one. The capacity of virus thermally inactivated at $37^\circ C$ to produce incomplete forms was further investigated by Paucker and Henle (1955a, b). Their results suggest that multiple infection with inactivated virus alone is sufficient to produce incomplete virus. In general, the presence of thermally inactivated virus in inocula resulted in preparations of lower ID$_{50}$/HA
Ginsberg (1954) and Horsfall (1954; 1955) evolved a hypothesis of incomplete virus formation based on the possibility that multiple infection results in cell damage or death due to virus toxicity and mature virus can no longer be completed in such conditions. This hypothesis seems difficult to sustain on the widely varying behaviour of different strains (Fazekas de St. Groth and Graham, 1954a). Henle and co-workers appear tacitly to accept the general idea of some form of intracellular interference, which is sometimes expressed as an "overloading" of host cells.

The nature of the chemical induction of incomplete virus, which gave rise to the receptor integrity hypothesis of Fazekas de St. Groth and Graham (1955) has been challenged. Liu, Paucker and Henle (1956) found that active virus was rapidly inactivated in vitro, in allantoic fluid containing periodate or periodate neutralized with glycerol, and this could account for the low ratio of yields. Schlesinger and Karr (1956) obtained similar results. Consequently, there seems to be nothing in conflict with the general notion that, when incomplete virus is occurring, it is associated with inocula large enough to infect every cell. Beyond this point however, no clear cut picture emerges of how or why incomplete virus occurs. Technical difficulties associated with the use of whole eggs have made quanti-
tative studies difficult. The widely held view that multiple infection is the critical factor derives support but not proof from these studies. Similarly, it is not possible to distinguish at present whether there is any trend in this association. Obviously it is important not only to know whether this effect is multiplicity dependent, but also whether a specific critical multiplicity is required. It is one of the objects of this section to reconsider this problem.

All quantitative studies of influenza virus multiplication in whole eggs have been plagued by both an uncertainty in multiplicity estimates and the considerable egg to egg variation in susceptibility to infection. Small pieces of surviving allantois cut from eggs provide a system suitable for infectivity titrations in which inter-egg variation is virtually non-existent (Fazekas de St. Groth and White, 1958b). An experimental system employing standard sized pieces of allantois cut from de-embryonated eggs is described here. The number of cells available in such pieces can be determined with reasonable accuracy from the figures of Cairns and Fazekas de St. Groth (1957). The actual experimental procedure consists of determining first cycle yields for varied inputs of virus into these bits. This type of experiment, as distinct from infectivity titrations is a direct means of determining the relation of yield to input. The overall object of this section is to see
how cells respond to infection under varying conditions of input; to see whether they behave as though single particles or more than one particle are needed to produce infection, and to see whether the formation of incomplete virus is dependent on a particular multiplicity of infection. The multiplication of normal virus in normal host cells can then be used as a standard of reference when considering situations where virus production is not the result of infection of cells by single infectious units.

Theoretically, if a cell will produce virus only when a certain number of particles are present, the response of yield to input can be predicted from the Poisson distribution, assuming that all cells behave in a similar fashion. If \( r + 1 \) particles per cell are necessary for it to yield, then the relation between the proportion \( P \) of cells yielding, and the multiplicity of infection \( x \), will be governed by the equation:

\[
P = 1 - e^{-x} \left( 1 + x + \frac{x^2}{2!} + \frac{x^3}{3!} + \frac{x^4}{4!} + \ldots \right)
\]

In the simplest case, where \( r = 0 \), \( P \) becomes equal to \( 1 - e^{-x} \).

Figure 1 shows the relation between \( \log_{10} x \) and \( \log_{10} P \) when the number of particles needed per cell to obtain yield varies from one to six. As the number of particles required increases, the slopes of the corresponding curves become much steeper.
Figure 1. The theoretical relation between input multiplicity and yield, when the numbers of particles per cell required for it to yield varies from one to six.
A curve closely resembling the single particle curve has been obtained for influenza haemagglutinin production in whole eggs (Cairns and Edney, 1952). Application of the same principle to the distribution of takes as a function of input in infectivity titrations similarly suggests that single infectious units are capable of initiating infection (Fazekas de St. Groth and Cairns, 1952; Fazekas de St. Groth and White, 1958b), although the slope of the curve obtained in whole eggs is significantly flatter than the theoretical and suggests the presence of a host factor tending to lower the probability of infection. Consequently, in an adequate test system the multiplication of standard influenza virus should approximate a one particle curve. Any flattening would be attributable to variable host susceptibility. However, conditions will later be described in which response curves of slope steeper than expected for single particles are encountered. Steeper slopes suggest collaboration between particles. An hypothesis based on the notion of collaboration or co-operation will be later developed to account for the appearance of incomplete virus.
1. Preliminary Experiments and Technical Procedures.

The Single Cycle Yield Experiment.

This type of experiment is used throughout the remaining chapters of this thesis. The experimental procedure is described in detail in the Appendix. It will be briefly described here.

Pieces of shell 1 sq. cm. in area with allantois attached were cut from eleven day old chick embryos. These bits were placed individually in cups of large Perspex trays. Appropriate dilutions of virus were added to the bits in 0.6 ml. volumes, using two egg pieces per dilution step. The trays were placed on a horizontal shaking machine operating in a constant temperature room at 35°C, and adsorption of virus was allowed for one and a half hours. Further adsorption of virus was prevented, and any surface bound, unadsorbed virus was released by the addition at this time of about 100 units of RDE, which was allowed to act for one hour. Two and a half hours after infection, the inocula were removed and the bits were rinsed with several changes of fresh medium. Finally 0.7 ml. of medium was added to each bit and the trays were returned to the shaker for another five to five and a half hours. At the conclusion of the experiment, the yields thus obtained
were titrated for haemagglutinin and infectivity.

Table 1 demonstrates the efficiency of uptake by bits as measured by haemagglutination, and regardless of input, is usually about 50%, as found for whole eggs (Cairns and Edney, 1952). Each value represents the mean obtained from twenty egg pieces, using the Influenza A strain MEL.

All subsequent multiplicity estimates consequently have been based on the assumption of 50% uptake.

Estimation of Multiplicity.

Carefully determined estimates of the allantoic cell numbers for eleven day old embryos (Cairns and Fazekas de St. Groth, 1957) reveal that there are $1.8 \times 10^7$ surface cells of the chorio-allantois covering an area of 55 sq. cm. Consequently, one sq. cm. bits contain about $3.3 \times 10^5$ surface cells. Allowing for an uptake of 50% of the inoculum, and for a factor of 0.15 $\log_{10}$ to correct $ID_{50}$ to infectious units, multiplicity estimates were routinely determined by subtraction of 0.45 $\log_{10}$ from the $ID_{50}$ $\log_{10}$ titre of the total input per bit. This correction has been included in columns of multiplicity listed in Tables.

Infectivity-Haemagglutinin ratios of yields.

Maximum values for the $ID_{50}/HA$ ratio are obtained when virus is obtained after small inocula. Fazekas de St. Groth and Cairns (1952) have defined a standard value for this ratio, where one agglutinating dose of influenza virus (the amount which
TABLE 1.

VIRUS UPTAKE.

The amount of virus taken up by cells in the standard single cycle yield experiment.

<table>
<thead>
<tr>
<th>Input AD's/bit</th>
<th>Residual AD's/bit</th>
<th>Adsorbed AD's/bit</th>
<th>Percentage Uptake</th>
</tr>
</thead>
<tbody>
<tr>
<td>100</td>
<td>50</td>
<td>50</td>
<td>50</td>
</tr>
<tr>
<td>58</td>
<td>34</td>
<td>24</td>
<td>41</td>
</tr>
<tr>
<td>26</td>
<td>12</td>
<td>14</td>
<td>54</td>
</tr>
<tr>
<td>13</td>
<td>5</td>
<td>8</td>
<td>61</td>
</tr>
<tr>
<td>6.5</td>
<td>2.5</td>
<td>4</td>
<td>62</td>
</tr>
</tbody>
</table>

Mean Percentage Uptake 54%.
gives partial agglutination with 0.25 ml 1% fowl red cells), consists of $10^{6.26 \pm 0.05}$ 50% infective units (ID$_{50}$) of virus. Virus preparations with values of this order fulfil the requirements for complete virus, whereas lower values are indicative of the presence of non-infectious, incomplete virus. However, regardless of the virus strain, the ID$_{50}$/HA ratios obtained from individual eggs vary over at least a ten to twenty-fold range (von Magnus, 1951a; Fazekas de St. Groth and Graham, 1954a). In experiments carried out entirely in egg pieces from one egg, the value of the ID$_{50}$/HA ratio considered as characteristic of complete virus for that particular egg was obtained from yields in the relatively low input range. This value was then used as a standard of comparison for comparing the ID$_{50}$ and haemagglutinin yields of all inputs, and as a point of reference for setting the scales of these values in figures. Infectivity titrations of yields were carried out by the method of Fazekas de St. Groth and White (1958a), and haemagglutinin titrations by the method of Fazekas de St. Groth and Graham (1955), except that a 5% suspension of fowl red cells was used for the latter, which is half the usual strength. In accordance with the standard ID$_{50}$/HA ratio value mentioned above, use of such half strength red cells in this case should result in complete virus ratios of the order of $10^{5.9}$. However, despite the finding of Fazekas de St. Groth and White (1958c),
that the sensitivity of infectivity titrations carried out in egg pieces is the same as in the orthodox allantoic infectivity tests, our experience has been that infectivity values, and consequently \(\text{ID}_{50}/\text{HA}\) ratios, obtained using this method are mostly about three-fold lower than when whole eggs are used. Determination of the \(\text{ID}_{50}/\text{HA}\) ratios for the yields of 35 standard virus preparations of MEL obtained from individual eggs harvested after 24 hr. incubation using these methods gave a standard value for the ratio of \(10^{5.45 \pm 0.27}\). The range of individual values varied between \(10^{4.81}\) and \(10^{5.94}\).

The Release Rate of Virus.

Cairns (1957), using the influenza A strain BEL, has found that there is a highly variable delay at some stage in the infection of allantoic cells which seems to be characteristic for each virus-cell encounter, and which is greatly reduced if the multiplicity per cell is increased. This is reflected in the much more rapid and uniform release of virus when large inputs are inoculated than when small inputs are used. In single cycle yield experiments which are terminated well before virus release is complete, this phenomenon has a marked effect. The nature of the phenomenon is demonstrated in the following experiment, using the strain MEL.

Groups of ten 15-day de-embryonated eggs were inoculated with dilutions of stock MEL in standard medium to give
approximate multiplicities of 20, 8, 2 and 0.25. Ninety minutes after inoculation the groups received 3200 units of RDE. The RDE was allowed one hour for receptor destruction, and then all eggs were rinsed three times, which was sufficient to ensure that any residual amount of inoculated virus was not enough to contribute significantly to the titres of the first samples. The fluid from each egg was removed at half-hourly intervals up until seven hours, and fresh medium replaced which contained 30 units of RDE. Haemagglutination titrations were carried out on each sample, and the manner in which virus is released is shown in Figure 2. There is a close similarity in the shape of these curves to those shown by Cairns (1957) for strain BEL. With large inputs, there is no appreciable delay and all cells begin to release virus simultaneously. This is shown by a high peak of release followed by gradual decline, and resembles the release rate of virus from a single cell (Cairns, 1952). With lesser inputs, this synchronization is gradually obliterated and so with small inputs, the distribution represents a wide scattering in the time of release by individual cells. Consequently, as the multiplicity decreases, fewer cells initially infected yield at a given time. Alternatively, for high inputs cells might appear to be yielding more virus than when singly infected. This will be considered in relation to single cycle yields.
Figure 2. The observed rate of production of haemagglutinin by MEL in de-embryonated eggs. The effect of increasing multiplicity on the synchronization of release time.
2. The Dose - Response Relation.

Three representative influenza virus strains were used in these experiments which are known to differ considerably in their capacity to produce incomplete virus, as shown in the "incomplete" virus gradient of Fazekas de St. Groth and Graham (1954a). Strain MEL represents those strains which show the greatest difference in ID$_{50}$/HA ratio between complete and incomplete stocks, the value for incomplete preparations being one hundred-fold or more lower. Strain BEL represents strains showing intermediate differences of the order of twenty-fold, and strain LEE represents those strains which apparently do not form any incomplete virus. Appropriate dilution series of each virus type were prepared, and single cycle yield experiments carried out.

The results of three typical experiments for strain MEL are listed in Table 2, and plotted in Figures 3, 4 and 5. Each individual experiment was performed in bits from one egg only. Input multiplicity estimates determined in the manner described above are only approximate, for some error is introduced both by small inter-egg variation in allantoic cell density and by variation in uptake. Values for haemagglutinin yields are plotted in log$_2$ units in the figures. The response of yield to input as seen from these experiments behaves in a uniform and reproduceable fashion. Maximum haemagglutinin
TABLE 2.

The formation of incomplete virus.

The relation of input multiplicity to infectivity and haemagglutinin yields for the strain MEL.

<table>
<thead>
<tr>
<th>Dilution of Stock Virus (log_{10})</th>
<th>Input Virus a</th>
<th>Multi-Log (log_{10})</th>
<th>Egg 1</th>
<th>Egg 2</th>
<th>Egg 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/100</td>
<td>7.13</td>
<td>14.6</td>
<td>4.7</td>
<td>1.42</td>
<td>5.59</td>
</tr>
<tr>
<td>1/200</td>
<td>6.83</td>
<td>7.2</td>
<td>4.5</td>
<td>1.35</td>
<td>5.22</td>
</tr>
<tr>
<td>1/400</td>
<td>6.53</td>
<td>3.6</td>
<td>4.5</td>
<td>1.35</td>
<td>5.47</td>
</tr>
<tr>
<td>1/600</td>
<td>6.23</td>
<td>1.8</td>
<td>4.2</td>
<td>1.27</td>
<td>5.62</td>
</tr>
<tr>
<td>1/1600</td>
<td>5.93</td>
<td>0.9</td>
<td>3.7</td>
<td>1.11</td>
<td>5.75</td>
</tr>
<tr>
<td>1/3200</td>
<td>5.63</td>
<td>0.45</td>
<td>2.6</td>
<td>0.79</td>
<td>5.91</td>
</tr>
<tr>
<td>1/6400</td>
<td>5.33</td>
<td>0.23</td>
<td>1.6</td>
<td>0.48</td>
<td>5.75</td>
</tr>
<tr>
<td>1/12800</td>
<td>5.03</td>
<td>0.12</td>
<td>0.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/25600</td>
<td>4.73</td>
<td>0.06</td>
<td>-</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Stock Virus titre 10^{8.75} ID_{50}/0.25 ml.
(b) 0.6 ml. volume.
(c) Estimated multiplicity, using 10^{5.5} as number of cells per bit, and subtracting 0.45 log_{10} from input titre to allow for adsorption, and conversion of ID_{50} to infectious units.
(d) Average yield from two egg bits.
**Figure 3.** The relation between input multiplicity and yield obtained for strain MEL. Closed circles represent the haemagglutinin yields; open circles represent the corresponding values for infectivity. ID$_{50}$ and haemagglutinin yields are compared by obtaining an ID$_{50}$/HA ratio from the low input range.
Figure 4. The relation between input multiplicity and yield obtained for strain MEL. Closed circles represent the haemagglutinin yields; open circles represent the corresponding values for infectivity. ID<sub>50</sub> and haemagglutinin yields are compared by obtaining an ID<sub>50</sub>/HA ratio from the low input range.
Figure 5. The relation between input multiplicity and yield obtained for strain MEL. Closed circles represent the haemagglutinin yields; open circles represent the corresponding values for infectivity. ID$_{50}$ and haemagglutinin yields are compared by obtaining an ID$_{50}$/HA ratio from the low input range.
yields for bits obtained from different eggs are virtually identical, and this in other experiments has constantly been the case, so that for large experiments it is possible to use bits from different eggs without introducing any effects due to differences of yield. These maximum haemagglutinin values are also sufficient for a range of lower values to be obtained, so that the nature of haemagglutinin response to input can be determined. Here again the behaviour of individual eggs is constant, and a curve of slope 45° can easily be fitted to the results, suggesting a single particle curve. However, while there is a general resemblance to the single particle curve of Figure 1, there is a discrepancy in the point at which maximum yields occur. In fact, the experimental curves appear to be releasing more virus when multiple infection occurs, so that yields are two-fold more than expected. This effect can be attributed to the synchronization of release which occurs with large inputs, although faulty multiplicity estimates could be blamed. That the effect is wholly due to synchrony can be demonstrated by holding such experiments for 24 hours with ample RDE to obtain total cell yields. In these circumstances the effect disappears, and the experimental curves come to fit closely to the predicted. There is no flattening, indicating that variable susceptibility does not count. Consequently, bits provide an adequate system for demonstrating the response of
haemagglutinin yield to input.

The relation of infective virus yield to input also shows a relatively constant and reproduceable behaviour. For small, sub-saturating inocula the virus released is wholly infectious. At an input roughly corresponding to one particle per cell, a difference between total and infective virus yields begins to appear which rapidly falls to a characteristic level. Once this level of infectivity is reached no further fall occurs regardless of input. From the theoretical curves of Cairns and Edney (1952), the decrease in the amount of active virus in the transitional region between complete and incomplete virus could be accounted for as the yields from the diminishing fraction of cells infected with only one particle. Consequently, there is a strong suggestion that the change from complete to incomplete coincides with the change from single to multiple infection.

Table 3 and Figures 6 and 7 represent the typical results obtained for strain BEL. There is a close similarity in behaviour to MEL, except that the maximum haemagglutinin yields for this strain appear to be about two-fold higher, and the ID_{50}/HA ratio difference between complete and incomplete are much less than for MEL. Again, however, incomplete virus formation appears to be multiplicity dependent, and the multiplicity at which the effect occurs is the same as for strain MEL, so that there is no evidence that strain BEL forms less
TABLE 3.

The relation of input multiplicity to infectivity and haemagglutinin yields for the strain BEL.

<table>
<thead>
<tr>
<th>Input Virus a</th>
<th>Egg 1</th>
<th>Egg 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution of Stock Virus</td>
<td>ID_{50}/bit</td>
<td>Multi-a</td>
</tr>
<tr>
<td>1/50</td>
<td>7.28</td>
<td>19.20</td>
</tr>
<tr>
<td>1/100</td>
<td>6.98</td>
<td>9.60</td>
</tr>
<tr>
<td>1/200</td>
<td>6.68</td>
<td>4.80</td>
</tr>
<tr>
<td>1/400</td>
<td>6.38</td>
<td>2.40</td>
</tr>
<tr>
<td>1/800</td>
<td>6.08</td>
<td>1.20</td>
</tr>
<tr>
<td>1/1600</td>
<td>5.78</td>
<td>0.60</td>
</tr>
<tr>
<td>1/3200</td>
<td>5.48</td>
<td>0.30</td>
</tr>
<tr>
<td>1/6400</td>
<td>5.18</td>
<td>0.15</td>
</tr>
<tr>
<td>1/12800</td>
<td>4.88</td>
<td>0.08</td>
</tr>
<tr>
<td>1/25600</td>
<td>4.58</td>
<td>0.04</td>
</tr>
</tbody>
</table>

(a) Stock Virus titre $10^{7.60}$ $ID_{50}/0.25$ ml.
(b) Stock Virus titre $10^{8.82}$ $ID_{50}/0.25$ ml.
(c) 0.6 ml. volume.
(d) Estimated multiplicity, using $10^{5.5}$ as the number of cells per bit, and subtracting 0.45 $\log_{10}$ from the input titre to allow for adsorption, and conversion of $ID_{50}$ to infectious units.
(e) Average yield from two egg bits.
Figure 6. The relation between input multiplicity and yield obtained for strain BEL. Closed circles represent the haemagglutinin yields; open circles represent the corresponding values for infectivity. ID\textsubscript{50} and haemagglutinin yields are compared by obtaining an ID\textsubscript{50}/HA ratio from the low input range.
Figure 7. The relation between input multiplicity and yield obtained for strain BEL. Closed circles represent the haemagglutinin yields; open circles represent the corresponding values for infectivity. ID$_{50}$ and haemagglutinin yields are compared by obtaining an ID$_{50}$/HA ratio from the low input range.
incomplete virus because it requires a higher critical number of particles per cell.

In general, the behaviour of the influenza B strain LEE is not as reproduceable in bits as that of the A strains, and the infectivity titres of yields tend to be very low. However, an adequate experiment for this strain is listed in Table 4 and Figure 8. Despite low values, there does not appear to be any difference in ID$_{50}$/HA ratio regardless of input; so that for this strain, there is no difference between large and small input.

ID$_{50}$/HA ratio differences for complete and incomplete virus in these experiments are not as prominent as in whole eggs. The average difference for MEL is about twenty-fold and for BEL about two to four-fold, while LEE does not show a difference. Despite smaller individual differences, the relative capacities of these strains to produce incomplete virus is the same as for whole eggs, and so the "incomplete" virus gradient exists also for egg pieces.

**The Dose Response of Incomplete Virus.**

Normal virus multiplying in bits behaves in the expected Poissonian fashion, and is very reproduceable. Burnet, Lind and Stevens (1955) have suggested that incomplete virus consists of grades of incompleteness, and on passage is capable of limited multiplication. They further suggest that this multi-
TABLE 4.

THE FORMATION OF INCOMPLETE VIRUS

The relation of input multiplicity to infectivity and haemagglutinin yields for strain LEE.

<table>
<thead>
<tr>
<th>Input Dilution of Stock Virus</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt;/bit&lt;sup&gt;b&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt;)</th>
<th>Multiplicity&lt;sup&gt;c&lt;/sup&gt;</th>
<th>HA&lt;sup&gt;d&lt;/sup&gt; log&lt;sub&gt;2&lt;/sub&gt;</th>
<th>HA&lt;sup&gt;d&lt;/sup&gt; log&lt;sub&gt;10&lt;/sub&gt;</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt;</th>
<th>ID&lt;sub&gt;50&lt;/sub&gt;/HA</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/20</td>
<td>6.47</td>
<td>8</td>
<td>4.3</td>
<td>1.29</td>
<td>4.60</td>
<td>3.31</td>
</tr>
<tr>
<td>1/40</td>
<td>6.17</td>
<td>4</td>
<td>4.2</td>
<td>1.26</td>
<td>4.70</td>
<td>3.44</td>
</tr>
<tr>
<td>1/80</td>
<td>5.87</td>
<td>2</td>
<td>4.3</td>
<td>1.29</td>
<td>4.70</td>
<td>3.41</td>
</tr>
<tr>
<td>1/160</td>
<td>5.57</td>
<td>1</td>
<td>2.8</td>
<td>0.85</td>
<td>4.30</td>
<td>3.45</td>
</tr>
<tr>
<td>1/320</td>
<td>5.27</td>
<td>0.5</td>
<td>2.0</td>
<td>0.60</td>
<td>4.35</td>
<td>3.75</td>
</tr>
<tr>
<td>1/640</td>
<td>4.97</td>
<td>0.25</td>
<td>-</td>
<td>-</td>
<td>3.55</td>
<td>3.55</td>
</tr>
<tr>
<td>1/1280</td>
<td>4.67</td>
<td>0.125</td>
<td>-</td>
<td>-</td>
<td>3.35</td>
<td>3.35</td>
</tr>
</tbody>
</table>

(a) Stock Virus titre 10<sup>7.39</sup> ID<sub>50</sub>/0.25 ml.
(b) 0.6 ml. volume.
(c) Estimated multiplicity, using 10<sup>5.5</sup> as the number of cells per bit, and subtracting 0.45 log<sub>10</sub> from the input titre to allow for adsorption, and conversion of ID<sub>50</sub> to infectious units.
(d) Average yield from two egg bits.
Figure 8. The relation between input multiplicity and yield obtained for strain LEE. Closed circles represent the haemagglutinin yields; open circles represent the corresponding values for infectivity. ID$_{50}$ and haemagglutinin yields are compared by obtaining an ID$_{50}$/HA ratio from the low input range.
plication is due to single particles, although the slopes of their curves are inadequate for this conclusion. An experiment of a rather preliminary nature will be recorded here on the multiplication of first passage incomplete virus prepared in whole eggs. The purpose of this experiment was to determine whether the multiplication of incomplete virus is due to single particles and if so what fraction is capable of producing first cycle yields. The behaviour of standard virus in bits is so uniform that the method can be used as a rough estimate of titre for unknown preparations by comparing the position of the dose-response curve obtained for the unknown with that of a preparation of known titre.

First passage incomplete MEL was prepared in whole eggs by the inoculation of undiluted allantoic fluid of a standard MEL preparation, having a ratio of $10^{6.30}$. The ID$_{50}$/HA ratio for the incomplete virus thus obtained was $10^{4.10}$, which corresponds well with the usual values obtained for this strain (Fazekas de St. Groth and Graham, 1954a). Dose-response curves were obtained in egg pieces in the usual way for both the standard and first passage preparations. The results have been plotted in Figure 9. Only the haemagglutinin response for standard virus is shown, and this behaves in the expected fashion. The relative positions of the yields of the complete and incomplete preparations were determined by making
Figure 9. The multiplication of incomplete virus. The response curves obtained for a standard virus preparation (closed circles) and an incomplete virus preparation (half-closed circles), following equivalent inputs of haemagglutinin. The broken line to the left indicates the estimated position of the response curve for the incomplete preparation, assuming that the residual infective virus fraction is the only virus present able to multiply.
inputs of haemagglutinin equivalent. Obviously, the nature of the haemagglutinin response provoked by incomplete virus is a slope of 45° and so it is Poissonian. Single particles would appear to be producing haemagglutinin. The magnitude of the displacement of this curve to the left of the standard curve gives an estimate of what fraction of the total population are multiplying. There is slightly more than a one log₁₀ difference, so that only one-tenth of the incomplete preparation is capable of multiplication. However, the ID₅₀/HA ratio of this preparation indicates that only one in every one hundred particles is infectious in the conventional sense, so that if these particles were the only ones able to produce yield, the position of the response curve would be as shown by the dotted curve on the extreme left of Figure 9. So that here again, there is a tenfold difference between the expected and found, which suggests that while only one in a hundred particles are capable of infinite multiplication, one-tenth of the population is capable of producing haemagglutinin. Consequently, the observations of Burnet, Lind and Stevens (1955) have been confirmed. The amount of infective virus produced by this first passage virus is very small, and does not show any particular trend, as shown by open circles in Figure 9. The ID₅₀/HA ratio of the second passage material is very low.

Further experiments such as these with incomplete virus
preparations offer a means of determining what fraction of these preparations are capable of multiplication.
DISCUSSION.

From the foregoing it can be seen that the determination of first cycle yields in bits with suitable strains is an easily handled and reproducible system. While technical limitations still hamper exact multiplicity determinations, most of the uncertainty involved in the use of whole eggs has been eliminated. The results for strains MEL and BEL reveal a reasonably clear cut association between incomplete virus formation and multiplicity. In the examples shown, a peak of infective virus production at or about a multiplicity of one particle per cell is shown, although in some experiments of this type this peak is not always prominent. In a similar experiment in whole eggs performed by Cairns and Edney (1952), there is also evidence for the existence of such a peak. The uniformity of the response and the multiplicity region in which it occurs suggests that there is a fundamental difference in the type of multiplication occurring when a cell is singly infected as opposed to when it is multiply infected. The results strongly suggest that not only is multiplicity a necessary condition for incomplete virus formation, but that there is a specific multiplicity, which appears to be two per cell, at which the effect occurs.

Two other observations on the nature of incomplete virus have been confirmed in this system. One is the existence
of a strain gradient in capacity to produce incomplete virus (Fazekas de St. Groth and Graham, 1953; 1954a), and the other is the existence of incomplete forms capable of limited multiplication (Burnet, Lind and Stevens, 1955). Fazekas de St. Groth and Graham (1953) felt that multiplicity alone could not be implicated as the prime cause of incomplete virus production because of the failure of strain LEE to produce incomplete virus under conditions of multiple infection. However, on passage, LEE does give rise to some incomplete virus (Fazekas de St. Groth and Graham, 1954a; von Magnus, 1954). As the results for incomplete producers such as MEL and BEL suggest that multiplicity is primarily involved, an explanation for the behaviour of LEE has to be sought. Two immediate possibilities from the available evidence spring to mind. The first is that under conditions of multiple infection for LEE, single particles are so asynchronous in starting time, that conditions are rarely attained where two particles begin multiplication at the same time, and so instead of collaboration, exclusion of the second particle occurs. In the strict sense of the word, multiple parents might not be achieved in these conditions. The other possibility is that under conditions of multiple infection, defective forms are being produced as with other strains, but these are capable of initiating a sufficient number of cycles of multiplication to score as positive in infectivity titrations.
The latter suggestion should be amenable to investigation by examining the dose-response behaviour of successive passages in the manner described for first passage MEL.

While the results confirm what appears to be a fairly universally held assumption - that multiplicity is necessary for incomplete virus production, they go further and suggest that not only is there a critical number, but its value is two. Various notions as to why multiplicity is important are of necessity extremely vague. The consensus of opinion seems to be that an "overloading" of cells is occurring. Certainly it seems that if this "overloading" is the result of competition for some essential substrate in short supply or the result of receptor destruction or even cell death, it is likely that the multiplicity effect would be relatively non-specific and variable. The constancy with which incomplete virus production occurs beyond a certain input level suggests that the effect is dependent on an interaction between the virus particles themselves. In effect, it seems likely that incomplete virus production is the result of particle interaction rather than the result of a non-specific effect. This notion will be further discussed later.

Ledinko, Bergs and Henle (1957) demonstrated the production of incomplete virus by the strain WSE in trypsinized chick embryo lung cells. In this case, beyond a multiplicity of one EID$_{50}$ per cell incomplete virus appeared. The nature
of the response curve obtained by these authors strongly suggests that these host cells either yield more virus on multiple infection or else classes of insusceptible cells exist which will only yield when they contain many virus particles. In addition, only about one in ten of the available cells was capable of supporting multiplication. Consequently, there are considerable quantitative uncertainties in these results. However, in view of the results reported here it is likely that multiple infection is inducing incomplete virus formation in these cells.

As an experimental system, the dose-response technique employed here provides a suitable means of seeking the existence of co-operative forms of multiplication, such as multiplicity reactivation.
The multiplication of influenza virus was investigated by performing single cycle yield experiments in pieces of surviving allantois of uniform size. This system provided a simple and reproducible means of determining the relation between input multiplicity and incomplete virus formation.

The existence of an incomplete virus gradient has been confirmed, using three well studied influenza virus strains. The results suggest that incomplete virus formation is multiplicity dependent. Two or more parental particles per cell usually result in incomplete virus formation. The notion is developed that incomplete virus formation is due to interaction between parental particles.

The existence of grades of incompleteness has also been confirmed. The nature of the response curve obtained suggests that some single incomplete virus particles are capable of further multiplication.
CHAPTER II.

MULTIPLICITY REACTIVATION.

INTRODUCTION.

Multiplicity reactivation was first reported by Luria (1947) for bacteriophage, when he found that two or more ultra-violet inactivated T-even phage particles, each by itself unable to reproduce, can sometimes co-operate to produce infective progeny if they happen to infect the same bacterial cell. This was interpreted as being the result of a genetic interchange of undamaged parts between the irradiated phages. This recombination theory of multiplicity reactivation was further developed by Luria and Dulbecco (1949). The rate of inactivation of bacteriophage exposed to ultra-violet light is a simple logarithmic function of dose of irradiation, and this "one-hit" inactivation process was considered to be the result of damage to discrete, self-contained genetic units. Inactivation of one such unit would inactivate the particle, but active virus would result if that damaged unit could be replaced by an undamaged unit of the same type from another particle by means of recombination under conditions of multiple infection. The probability that a multiply infected bacterium contains a complete set of undamaged units should simply be a
function of the number of units in the particle, the proportion of units damaged by irradiation, and the number of particles per bacterium. This is the simplest theory which could account for multiplicity reactivation, and describes the most efficient means by which such unit substitution could occur. While the experiments of Luria and Dulbecco (1949) agreed quite well with the predictions, closer examination of the situation by Dulbecco (1952) revealed that at low levels of survival for the bacteriophage T2, more reactivation was occurring than could be accounted for by the proposed theory. As no modification of the recombination theory could at the time be found to account for this discrepancy, it appeared to be disproved. Cairns and Watson (1956), by making allowance for variation in bacterial cell size, produced theoretical curves based on the recombination theory in good agreement with the experimental points of Dulbecco. However, variation in host cell size is not the factor responsible. Modifications of Luria's original formulation have now, however, been produced which reconcile the experimental observations with the recombination theory (Harm, 1956; Baricelli, 1956). These modifications imply that two types of unit exist of very different sensitivity to inactivation. Baricelli (1956) suggests that the subunits of the genetic portion of the phage are numerous and are part of a continuous genetic structure, rather than independent. Recombination
between undamaged parts of these parental structures occurs, but these subunits have a probability of inactivation which is negligible when compared to the probability of inactivation of the whole phage. So in addition, particularly sensitive units, the "vulnerable" centres exist, which are necessary for the replication of the genetic material and are located in either the genetic or somatic structures of the virus. One undamaged copy of each type of vulnerable centre is also essential for multiplicity reactivation.

This model accounts for the type of multiplicity reactivation encountered with the T-even bacteriophages. On the other hand, Bernstein (1957) has found that Vi-phage II of Salmonella typhi undergoes a type of multiplicity reactivation which closely fits the Luria and Dulbecco model, and there appears to be about four ultraviolet sensitive units in this phage. The evidence suggests then that for bacteriophage, multiplicity reactivation is a manifestation of genetic recombination, and that the complexities of this reactivation are associated with the nature of the lesions produced by irradiation.

Henle and Liu (1951) claimed to have demonstrated multiplicity reactivation for influenza virus. Their evidence was based solely on the fact that the appearance of progeny was much more rapid following large inocula of UV inactivated
virus preparations than when dilutions of active virus alone equivalent to the residual activity of the UV preparations were used as inocula. This was a completely unsuitable experimental system for seeking multiplicity reactivation. The appearance of progeny in such a system is affected considerably by the presence of host cell receptors. If small amounts of active virus are followed by totally inactivated virus or RDE, cell receptors are destroyed, virus release is rapid, and results indistinguishable from those of Henle and Liu are obtained (Cairns, 1955). Consequently, whether or not multiplicity reactivation was occurring, it could not be demonstrated by this means. Drake (1958) claimed that poliovirus undergoes multiplicity reactivation, but adaptation of the recombination theory of Luria and Dulbecco (1949) to this situation revealed that the rate of disappearance of yielders as a function of UV dose was close to the rate of inactivation of the virus itself, and an estimate of the number of units comprising the virus genome was found to have a non-integer value which was less than two. Consequently, the evidence for multiplicity reactivation with this virus is not very strong. Whether the functional behaviour of virus ribonucleic acid, the sole nucleic acid component of both these viruses, is in any way analogous to that of phage DNA is not known. However, influenza virus undoubtedly undergoes genetic
interaction, but the situation is very complex and difficult to analyse. If multiplicity reactivation can be demonstrated for influenza virus, then there is a means at hand whereby this functional behaviour, and its relation to genetic recombination can be examined. In considering any reactivation process however, it is necessary to know whereabouts in the virus particle the inactivating lesions are occurring. It is implicit in considerations of multiplicity reactivation, that these lesions are occurring in the virus genome. The evidence that the inactivating effect produced by UV light occurs in the phage genome is based on action spectra of UV light for phages, which indicate that photons are adsorbed by purine and pyrimidine residues of the phage DNA (Gates, 1934; Fluke and Pollard, 1949; Franklin et al., 1953). Also, genetic studies by Doermann et al. (1955) and Epstein (1958) suggest that the UV damages affecting recombination are located in the genetic structures of bacteriophage. The action spectrum of UV light for influenza virus infectivity as reported by Holleander and Oliphant (1944), Tamm and Fluke (1950), Powell and Setlow (1956), show a pattern characteristic of that observed for nucleic acid. Some, but not all of the UV lesions produced in bacteriophage by UV irradiation are potentially reversible for they can be repaired by a substance generated in the bacterial host cell by visible light. This
repair process has been called photoreactivation (Dulbecco, 1950) and is not multiplicity dependent.

In this section, evidence for reactivation following inactivation by UV irradiation of influenza virus is presented, using the experimental system described in the preceding section, and this reactivation is shown to be multiplicity dependent. Such inactivated preparations do not contain some virus which is capable of limited cycles of multiplication, as has been suggested by Fazekas de St. Groth (1958).
The Inactivation of Influenza Virus by Ultraviolet Irradiation.

The irradiation of influenza virus stocks was carried out as follows. Ten ml. amounts of dialysed allantoic fluid containing virus were exposed for various intervals of time in open Petrie dishes, 7 cm. in diameter, to a Phillips 15 W TUC germicidal lamp, at a distance of eight inches. The layer of fluid in these dishes was about one mm. in thickness, and was subjected to continuous rocking during irradiation, to ensure mixing. More than 95% of the output from this lamp was of wave length 2537A. The residual infectivity of each preparation after irradiation was determined by the method of Fazekas de St. Groth and White (1958a). Figure 1 represents a typical inactivation curve, obtained with strain MEL. Identical curves have also been obtained for strains BEL and LEE. The inactivation of influenza virus by ultraviolet light shows a uniform exponential decrease, indicating a "one-hit" process. Sometimes however, a decided change of slope occurs at relatively low levels of survival, as shown in Figure 2. This curve bears a strong resemblance to the inactivation curve obtained for bacteriophage T4 (Luria and Dulbecco, 1949), in which change of slope is indicative of multiplicity reactivation. While multiplicity reactivation could no doubt be
Figure 1. The inactivation of influenza virus infectivity by ultraviolet irradiation. \( \frac{V}{V_0} \) is the proportion of active virus after irradiation. \( r = \log_e \frac{V_0}{V} \) and is the average number of lethal hits per virus particle. The doses are expressed in seconds of exposure.
Figure 2. The inactivation of influenza virus infectivity by ultraviolet irradiation. $\frac{V}{V_0}$ is the proportion of active virus after irradiation. $r = \log_e \frac{V_0}{V}$ and is the average number of lethal hits per virus particle. The doses are expressed in seconds of exposure. The deviation for high doses in the curve which is encountered in some virus preparations is shown. The broken line represents extrapolation from the logarithmic portion of the curve.
contributing to this slope alteration for influenza virus, the general lack of regularity in its occurrence suggests some other cause. It might just as well indicate the existence of a small fraction of virus in a "protected" state. The virus stocks used in these experiments are harvested as early as is compatible with high titre. Often in such preparations, as much as 50% of the total virus is inhibitor bound and cannot be detected by haemagglutination. This bound virus can be sedimented by centrifugation at a temperature of 2-4°C at 850 g for 45 minutes. If such allantoic fluid preparations are incubated at 37°C with or without RDE for 30 minutes, most of this bound virus is recovered. Some such inhibitor bound virus may be in a relatively protected state, and would provide an explanation for this slope alteration. The inactivation curve for virus infectivity shown by Henle and Henle (1947) also shows a decided kink at low levels of survival.

However, in the majority of cases, inactivation of infectivity is a regular "one-hit" process. This implies that the absorption of a single lethal hit is sufficient to inactivate an influenza virus particle. In Figures 1 and 2, the proportion of residual active virus $\frac{V}{V_0}$ is plotted against time of exposure, in seconds. If for a given irradiation dose, the average number of effective hits per particle is $r$, the proportion of active to total virus will be $e^{-r}$ i.e. $\frac{V}{V_0} = e^{-r}$.
Consequently, from the determined inactivation rate, one can obtain the average number of lethal hits per particle for a particular irradiation dose as \( \log_e \frac{V}{V_0} \) (Luria and Dulbecco, 1949). The calculated hit rate is also shown in Figures 1 and 2. Irradiated preparations will subsequently be referred to by the average number of hits per particle they have received.

**The Dose - Response Behaviour of Irradiated Virus.**

1. **Preliminary Experiments.**

The experimental system employed was identical to that described in Chapter I for obtaining dose-response curves for standard virus preparations.

Irradiated virus preparations of the strains MEL, BEL and LEE were used. These were obtained by irradiating 10 ml. volumes of virus from the one virus stock for various intervals of time. The hit rate of each preparation was determined in the manner described above. These preparations could be snap-frozen in dry ice - alcohol mixtures and stored at \(-72^\circ \text{C}\) without loss of activity. For standardizing the input multiplicities of irradiated preparations, single cycle yield experiments were always performed at the same time with unirradiated virus from the same stock.

We have already discussed in the previous chapter on incomplete virus formation, the type of relation between input
and first cycle yield to be expected when cells can only be made to yield by a co-operative action between several particles infecting them. The distinction between such co-operative action and the normal infective process is shown in Figure 1 (Page 71).

A typical experiment using two irradiated preparations of strain MEL, having an average of 5.6 and 8.2 hits per particle respectively is shown in Table 1 and Figure 3. The standard virus control had an infectivity titre of \(10^{7.55}\) ID\(_{50}/0.25\) ml., and only the haemagglutinin yields produced by this preparation were determined. Multiplicities for the irradiated preparations were calculated from the infectivity of the preparation before irradiation. The columns of Table 1 showing ID\(_{50}\) input per hit for the irradiated virus preparations represent the amount of residual infective virus present, and which by comparison with the ID\(_{50}\) inputs of standard virus necessary to produce yield seems unlikely to account for the yields produced.

The total haemagglutinin yields produced in bits by irradiated preparations are not as high as for normal virus preparations, and the range in which haemagglutinin can be detected is not therefore sufficient to reveal the nature of the response curve. However, the determination of infective virus yields for all inputs strongly suggests that below a
### TABLE 1.

**The Multiplication of Influenza Virus Inactivated by Ultraviolet Irradiation.**

<table>
<thead>
<tr>
<th>Standard Virus a</th>
<th>5.6 hit UV Virus a</th>
<th>8.2 hit UV Virus a</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Input</strong></td>
<td><strong>Yield</strong></td>
<td><strong>Input</strong></td>
</tr>
<tr>
<td>Diln. of ID$<em>{50}$/bit stock virus (log$</em>{10}$)</td>
<td>Multiplicity</td>
<td>Diln. of ID$<em>{50}$/bit stock virus (log$</em>{10}$)</td>
</tr>
<tr>
<td>$1/10$</td>
<td>6.93</td>
<td>8</td>
</tr>
<tr>
<td>$1/20$</td>
<td>6.63</td>
<td>4</td>
</tr>
<tr>
<td>$1/40$</td>
<td>6.33</td>
<td>2</td>
</tr>
<tr>
<td>$1/80$</td>
<td>6.03</td>
<td>1</td>
</tr>
<tr>
<td>$1/160$</td>
<td>5.73</td>
<td>0.50</td>
</tr>
<tr>
<td>$1/320$</td>
<td>5.43</td>
<td>0.25</td>
</tr>
<tr>
<td>$1/640$</td>
<td>5.13</td>
<td>0.13</td>
</tr>
<tr>
<td>$1/1280$</td>
<td>4.83</td>
<td>0.06</td>
</tr>
</tbody>
</table>

(a) Standard Virus titre $10^{7.55}$ ID$_{50}$/0.25 ml.; 5.6 hit UV virus titre $10^{5.10}$ ID$_{50}$/0.25 ml.; 8.2 hit UV virus titre $10^{4.05}$ ID$_{50}$/0.25 ml.

(b) Multiplicity determined for standard virus by subtracting $0.45$ log$_{10}$ from total input.

(c) Multiplicity determined for irradiated virus on the basis of its original infectivity.
**Figure 3.** The multiplication of ultraviolet irradiated influenza virus. Closed or half-closed circles represent haemagglutinin yields. Open circles represent yields of infective virus.
certain input level of irradiated virus, the only virus being produced is fully complete virus. This is indicated by the fact that at about an average multiplicity of one particle per cell, the ID_{50}/HA ratios of the yields obtained for either of these irradiated preparations are in the normal range for this system, whereas at higher multiplicities incomplete virus formation is definitely occurring. If one assumes that below this critical multiplicity, only complete virus is being produced, then the slope of the response curve can be easily obtained. Comparison of these slopes in Figure 3 with either the control, or the theoretical curves shown in Figure 1 of Chapter I, indicate the existence of a multicomplex curve. This is presumptive evidence for multiplicity reactivation.

Several subsidiary experiments were carried out to check the validity of this assumption.

First, if the low infectivity yields at low input multiplicities truly reflect the total yield, then if the response could be detected through a greater range by haemagglutination, it should behave in an identical fashion. On the other hand, the occurrence of any incomplete forms at these low levels could not be detected, but if present they could alter considerably the steepness of the response curve. Accordingly, the capacity of an irradiated preparation to produce haemagglutinin in de-embryonated eggs was determined,
for this system has a much greater haemagglutinin range.

The following experiment was performed. Two-fold dilution series of a standard virus preparation and an 8.2 hit UV preparation were prepared. Two 15-day old de-embryonated eggs were used per dilution step of each. After de-embryonation, these eggs received 2 ml volumes of SM and were rotated on a rotating machine in a 35°C constant temperature room for one hour prior to receiving virus. Then this fluid was removed and replaced by 2 ml volumes of SM with the appropriate virus dilution. One and a half hours after infection, 3200 units of RDE was added to each egg. At two and a half hours, the inocula were removed, and each egg was rinsed sufficiently often to remove any trace of unadsorbed virus. Haemagglutination titrations on the original inocula revealed that between 50-75% of the virus had been adsorbed. The experiment was terminated at 7 hours, and the content of each egg assayed for haemagglutinin. These titres are listed in Table 2 as the geometric mean titre for each dilution. Multiplicity estimates are based on a value of 3.2 x 10⁷ surface cells for such eggs (Cairns and Fazekas de St. Groth, 1957) and an uptake of 50%, but variation in egg size and uptake is such that these multiplicities are only very approximate. The results are plotted in Figure 4. The close parallel between the results obtained in de-embryonated eggs and in bits suggests
TABLE 2.

THE MULTIPLICATION OF ACTIVE AND ULTRAVIOLET IRRADIATED INFLUENZA VIRUS PREPARATIONS IN DE-EMBRYONATED EGGS.

<table>
<thead>
<tr>
<th>Standard Virus a</th>
<th>Input</th>
<th>Yield</th>
<th>8.2 hit UV Virus a</th>
<th>Input</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution ID&lt;sub&gt;50&lt;/sub&gt;/egg Multi-b plicity HA log&lt;sub&gt;2&lt;/sub&gt;</td>
<td>Dilution ID&lt;sub&gt;50&lt;/sub&gt;/egg Multi-b plicity HA log&lt;sub&gt;2&lt;/sub&gt;</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/5 8.50 3.6</td>
<td>8.8</td>
<td>0 5.60 16</td>
<td>7.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/10 8.20 1.8</td>
<td>8.6</td>
<td>1/2 5.30 8</td>
<td>6.8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/20 7.90 0.9</td>
<td>8.7</td>
<td>1/4 5.00 4</td>
<td>7.0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/40 7.60 0.45</td>
<td>7.4</td>
<td>1/8 4.70 2</td>
<td>5.3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/60 7.30 0.22</td>
<td>7.0</td>
<td>1/16 4.40 1</td>
<td>1.9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/160 7.00 0.11</td>
<td>6.5</td>
<td>1/32 4.10 0.50</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/320 6.70 0.6</td>
<td>5.7</td>
<td>1/64 3.80 0.25</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/640 6.40 0.3</td>
<td>3.5</td>
<td>1/128 3.50 0.12</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/1280 6.10 0.2</td>
<td>2.4</td>
<td>1/256 3.20 0.06</td>
<td>0</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1/2560 5.80 0.1</td>
<td>1.4</td>
<td>1/512 2.90 0.03</td>
<td>0</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

(a) Standard Virus titre 10<sup>8.3</sup> ID<sub>50</sub>/0.25 ml.; irradiated virus 10<sup>4.7</sup> ID<sub>50</sub>/0.25 ml.

(b) Multiplicity based on 10<sup>7.51</sup> cells/egg, and 50% adsorption of inoculum.
Figure 4. The multiplication of active and UV irradiated virus in de-embryonated eggs. The steepness of the haemagglutinin response curve shown by the irradiated preparation indicates that reactivation is occurring.
that the assumption that rising infectivity values in the relatively low input range adequately measure total virus yield, and so give a reliable estimate of the response curve, is correct.

Secondly, although the amount of residual active virus present in the irradiated preparations appears to be insufficient to contribute significantly to the results, in the high dilution range particularly it may be exerting an effect. For this reason, sufficient active virus was added to the 8.2 hit UV preparation of Table 1, to raise its residual infectivity titre ten-fold, i.e. from $10^{4.1}$ to $10^{5.1}$ ID$_{50}$/0.25 ml. This now becomes equivalent to the 5.2 hit preparation. Single cycle yield for this virus is shown in Table 3 and Figure 5. This curve is indistinguishable from that produced by the unsupplemented material in either position or slope of the response curve. Consequently, the residual active virus of such preparations plays a negligible part in the production of virus, and the effect is wholly attributable to the inactivated virus. Both the multiplicity conditions and the steepness of the response curve indicate that this is multiplicity reactivation.

2. **The Nature of the Reactivation Process.**

The model proposed by Luria and Dulbecco (1949) is the simplest means of describing reactivation, and so the
TABLE 3.

THE EFFECT OF THE ADDITION OF ACTIVE VIRUS ON THE
MULTIPLICATION OF ULTRAVIOLET IRRADIATED VIRUS

<table>
<thead>
<tr>
<th>Dilution of Stock Virus</th>
<th>Input ID_{50}/bit &amp; HA-log_{10}</th>
<th>Yield ID_{50}/log_{10}</th>
<th>HA-log_{2}</th>
<th>HA-log_{10}</th>
</tr>
</thead>
<tbody>
<tr>
<td>1/6</td>
<td>4.70 16</td>
<td>3.5 1.05</td>
<td>5.30</td>
<td></td>
</tr>
<tr>
<td>1/12</td>
<td>4.40 8</td>
<td>3.2 0.96</td>
<td>4.90</td>
<td></td>
</tr>
<tr>
<td>1/24</td>
<td>4.10 4</td>
<td>2.9 0.88</td>
<td>4.70</td>
<td></td>
</tr>
<tr>
<td>1/48</td>
<td>3.80 2</td>
<td>2.0 0.60</td>
<td>5.00</td>
<td></td>
</tr>
<tr>
<td>1/96</td>
<td>3.50 1</td>
<td>1.0 0.30</td>
<td>5.40</td>
<td></td>
</tr>
<tr>
<td>1/192</td>
<td>3.20 0.5</td>
<td>0 0</td>
<td>4.88</td>
<td></td>
</tr>
<tr>
<td>1/384</td>
<td>2.90 0.25</td>
<td>0 0</td>
<td>4.45</td>
<td></td>
</tr>
<tr>
<td>1/768</td>
<td>2.60 0.13</td>
<td>0 0</td>
<td>3.35</td>
<td></td>
</tr>
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Sufficient active virus was added to an 8.2 hit UV preparation to raise its residual infectivity titre tenfold, i.e. from $10^{4.1}$ to $10^{5.1}$ ID_{50}/0.25 ml.

(a) Multiplicity determined on the basis of the pre-irradiation titre of this preparation.
Figure 5. The effect of the addition of active virus on the multiplication of UV irradiated virus. Closed or half-closed circles indicate haemagglutinin yields, while open circles represent infectivity values.
behaviour of irradiated influenza virus will be considered in relation to the predictions of this theory. Following Luria and Dulbecco, a number of basic assumptions about the nature of the virus genome are necessary. The first is that each virus particle consists of a number of "units" $n$, each capable of being hit when exposed to UV irradiation. Since one effective hit is sufficient to inactivate a virus particle, inactivation of one unit makes the virus incapable of reproducing itself. Secondly, it is assumed that all units are of equal sensitivity, and finally that active virus cannot be produced in a cell unless the infecting particle or particles taken as a group, contain at least one copy of each unit in a non-lethal form. From these assumptions, the fraction of cells, $z$, in the total population which receive a full complement of active units can be described by the following expression:

$$z = \sum_{k=1}^{\infty} \frac{m^k e^{-m}}{k!} \left[ 1 - (1 - e^{-r/n})^k \right]^n$$

where $m$ is the average number of particles adsorbed per cell, $k$ is a particular multiplicity class of particles per cell for any input, $r$ is the average number of hits per particle, and $n$ is the number of units per virus particle.

As $r$, $m$ and $z$ can be determined experimentally, it is possible to see whether the dose-response curves obtained
in any way fit the "unit" model, by comparing them with theoretical curves obtained for various values of n. An additional requirement of this theory is knowledge of the distribution of particles among cells. If cells are relatively uniform in size, this distribution will be Poissonian. However, any marked lack of uniformity will modify the distribution. In considering the response of influenza in allantoic cells, the effect of any possible variation in size of the allantoic facet of individual cells has to be considered.

Figure 6 represents the relative frequency of cells differing in allantoic facet area for the allantois, obtained from the data of Cairns (unpublished). Assuming a Poisson distribution of influenza particles over the allantoic surface, a modified distribution for a number of multiplicity values has been determined, taking into account this variation in cell surface area. A comparison of the values obtained for this modified distribution with corresponding values of the Poisson distribution is shown in Table 4. Clearly, if the Poisson distribution were assumed to hold in this case, the number and frequency of multiply infected cells would be underestimated. The effects of this modified distribution on the occurrence of multiplicity reactivation will be considered later, but in general its effect is much greater if
Figure 6. The relative frequency of cells differing in allantoic facet area in the allantois, obtained from the data of Cairns (unpublished) and Cairns and Fazekas de St. Groth (1957).
### TABLE 4.

THE EFFECT OF VARIATION IN ALLANTOIC FACET AREA ON THE DISTRIBUTION OF PARTICLES AMONGST CELLS. *

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| P. = Normal Poisson Distribution. M. = Distribution of Particles among allantoic cells. |
| m = Average multiplicity per cell. |
* The modified distribution was obtained with the aid of the various classes of relative cell surface area shown in Figure 6. For each of these cell classes, an actual average multiplicity was calculated for any particular overall average multiplicity. The proportion of cells within each of these classes then showing a particular multiplicity (k) was calculated using the general term of the Poisson distribution $m^k e^{-m}$. For each value of k, a grand total was obtained and these are listed in the table in comparison to normal Poisson values.*
the reactivating particles consist of many rather than a few units.

Experimental response curves were obtained for a number of irradiated preparations of each of the strains MEL, BEL and LEE to determine their relation to each other, and to see how they fitted the predictions of the Luria and Dulbecco model. Figure 7 shows two such curves for the strain MEL. The slopes of these curves were obtained by using haemagglutinin values in the upper part to avoid complications due to incomplete virus formation, and infectivity values in the lower part, where haemagglutinin could not be determined. This figure shows the response of preparations which have received on the average 8.2 and 16.4 irradiation hits per particle. All the infectivity values obtained are included to show that in the upper part of the curve, incomplete virus formation is occurring.

Figure 8 shows another series of MEL preparations, and in this case only those values for either haemagglutinin or infectivity are shown which contribute to the determination of slope. As the irradiation dose increases, the number of particles per cell required to produce haemagglutinin increases, but in addition, as is seen from Figures 7 and 8, the ability of these irradiated particles to produce fully infectious virus is progressively lost. So, at low irradiation doses, the virus
Figure 7. The multiplication of two UV irradiated MEL preparations, showing multiplicity reactivation. Half-closed circles represent haemagglutinin yields, while open circles represent infectivity values.
Figure 8. The multiplication of three UV irradiated MEL preparations, showing multiplicity reactivation. Closed circles represent the haemagglutinin response of unirradiated influenza virus. Half-closed circles represent the haemagglutinin yields of irradiated preparations. Only those infectivity values (open circles) which indicate the slope of the response curves are included.
produced by reactivation is normally fully complete until almost maximum yield is appearing. However, as the irradiation dose increases, the level to which infectious virus values rise before incomplete virus formation is encountered progressively declines. So that in Figure 8, although the 25.9 hit preparation produced haemagglutinin at sufficiently high inputs of irradiated virus, it was not possible to detect any infectious virus being produced at all. So it seems that this virus is all incomplete, and the slope of the response curve for this preparation had to be guessed; this is shown by the broken line of similar slope to the other response curves. It seems that as irradiation dose increases, the progeny produced by reactivation become progressively more incomplete.

The maximum yields obtained following reactivation are never as great as those obtained with active virus, as is also shown in Figure 8.

Figure 9 shows a family of reactivation curves obtained with strain BEL. Here again, only those infectivity values which give an indication of the slope are shown. For any particular reactivating preparation, these infectivity values represent the highest level to which infectivity rises, so that all further yield is incomplete. The behaviour of strain BEL, both in the relative positions of the response curves for different irradiation doses, and in the way
Figure 9. The multiplication of four UV irradiated BEL preparations showing multiplicity reactivation. The response of unirradiated virus (closed circles) is also included. Half-closed circles represent the haemagglutinin yields for the irradiated preparations. Only those infectivity values (open circles) which give an indication of slope are shown.
progressive incompleteness is occurring, is very similar to that of strain MEL. Once again, maximum yields are invariably lower than the control, which is also shown. It is not clear why these reactivation curves flatten before reaching what is normally the maximum yield for this system. It may be that under conditions of reactivation, the total yield of progeny particles per cell is reduced. Alternatively, at high inputs some surface effect, such as receptor competition or destruction may be reducing the effective number of parental particles able to co-operate. However, this reduction in yield is a constant feature of reactivation.

Figure 10 shows the behaviour of a single irradiated LEE preparation which had received an average of 6.3 hits per particle, compared with the behaviour of an unirradiated preparation. This strain is reactivating in a similar fashion to the two other strains, and the production of incomplete virus is also occurring.

These experimental results show the way in which reactivation is occurring. It remains to be determined whether the slope and position of these curves in any way fits the predictions of the Luria and Dulbecco model. Consequently, a series of values for n, the number of units, was substituted into the equation shown above, and groups of curves for these values of n were calculated for various appropriate irradiation
**Figure 10.** The multiplication of active and UV irradiated LEE preparations. Half-closed circles represent the haemagglutinin yields obtained for the irradiated preparation. Open circles indicate the infectivity values.
doses. These curves were obtained by using the normal Poisson distribution instead of the modified distribution shown in Table 4, because for high hit rate preparations the positions of the experimental curves is such that multiplicities much greater than six are encountered, and the calculation of modified multiplicity values for the allantois at these higher values is extremely laborious. A number of values of n were tested. Figure 11 shows a group of theoretical curves for hit rates of 4, 8, 12, 16 and 26 per particle, assuming that influenza virus particle consists of six units. There is a considerable similarity in the position and spacing of these curves to those obtained experimentally. Figure 12 shows the same theoretical curves, but only for hit rates 8 and 16, in relation to the experimental points obtained for two MEL preparations, which had received 8.5 and 17.2 hits respectively, and which are taken from Figure 8. Here, the correspondence between the expected reactivation for a particle of six units and the reactivation encountered experimentally is very close. The results obtained for strain BEL (Figure 9) also approximate these theoretical curves, although in position they are slightly too far to the right. It is quite likely though that this is due to a faulty estimate of multiplicity.

The effect of the variation in size of the allantoic facets of allantoic cells has to be considered, however, The
Figure 11. The relation between the proportion of cells yielding and the multiplicity of infection for five irradiation doses, assuming that each particle consists of six irradiation sensitive units, obtained from the expression

\[ z = \sum_{k=1}^{\infty} \frac{m^k e^{-m}}{k!} \left[ 1 - (1 - e^{-r/n})^k \right]^n \]

where \( m \) is the average number of particles adsorbed per cell, \( k \) is a particular multiplicity class of particles, \( r \) is the average number of hits per particle, and \( n \) is the number of units per virus particle. Curves shown for the following values of \( r = 4, 8, 12, 16 \) and 26.
Figure 12. Theoretical curves obtained from Figure 11 for hit rates of 8 and 16, shown in relation to the experimental points obtained for two MEL preparations which had received 8.5 and 17.2 hits. Broken lines indicate the theoretical curves.
theoretical curves for six units and for two of the lower hit rates, namely eight and twelve, were redetermined using the distribution shown in Table 4, and these were compared to those previously obtained for these values with the Poisson distribution. Their relative positions are shown in Figure 13. Obviously, if the value of \( n \) is six, the effect of variation in host cell size is slight, although it would result in rather more reactivation than would be expected on the basis of the Poisson distribution alone. However, the range of this effect is well within the experimental uncertainty of the test system. So it does not seem to be necessary to make any correction for this effect, and Figure 12 is quite suitable as a standard of reference.

The theoretical curves obtained for \( n \) equal to six appear to be much closer to the experimental curves than those obtained for any other value of \( n \). However, the curves corresponding to five or seven units are not very different, and the quantitative uncertainties of the experimental system are such that no absolute distinction could be drawn between 5, 6 or 7, as the value of the number of units. However, six appears to be the closest fit. Any value of \( n \) beyond this range of values however, gives theoretical curves quite distinct in position from those obtained experimentally.
Figure 13. The effect of variation in size of the allantoic facets of allantoic cells on the position of theoretical curves. Solid lines indicate the curves obtained with the Poisson distribution; broken lines indicate the curves obtained with the modified distribution shown in Table 4, for values of $r = 8, 12$. 
DISCUSSION.

The results presented in this section indicate that UV irradiated influenza virus particles are capable of undergoing multiplicity reactivation. This is the first adequate demonstration of its occurrence for this virus. In fact it lays some claim to being the first clear-cut demonstration of the phenomenon for any virus whose genetic material is ribonucleic acid. Drake (1958) produced data on the multiplication of irradiated poliovirus which he claimed demonstrated multiplicity reactivation. However, in this case, the survival of multi-complexes does not fit the Luria and Dulbecco model, and it seems doubtful whether this survival can be distinguished from the survival of unirradiated virus. An added complication is the fact that the type of host cell used responded to infection in a way which suggests that classes of variable susceptibility exist. Even when multiplicity is fairly high, it is quite conceivable that the proportion of cells yielding virus may rise with rising input, not because co-operation is occurring between the entering, inactivated particles but because the probability of infecting the less susceptible cells in the population only becomes high when the input of residual infective virus into those cells also becomes high; in other words, the rising yield above expectation when multiplicity is increased is due to pulling
in the insusceptible cells, rather than ensuring that susceptible cells receive a full complement of intact sub-units. In the face of these difficulties the case for multiplicity reactivation by poliovirus is not strong.

As previously mentioned, Henle and Liu (1951) attempted to demonstrate the existence of the phenomenon for influenza virus. However, although they used irradiated preparations which were quite capable of multiplicity reactivation, and while this effect no doubt contributed to the results they obtained, their experimental approach was unsuitable. Their results were confounded with receptor destruction and rapid virus release (Cairns, 1955), an effect which had previously been used for obtaining one-step growth curves (Henle, Henle and Rosenberg, 1947).

A necessary requirement for the demonstration of multiplicity reactivation is that co-operative interaction between irradiated particles to produce live progeny is essential. This is clearly shown for influenza virus by the slope and position of curves which represent the response to input of irradiated virus. These curves fit the Luria and Dulbecco model reasonably well, with the most likely value for the number of units involved being six. It is not necessary to invoke at the moment the modifications of the simple recombination theory produced by Hann (1956) or Baricelli (1956)
needed to deal with multiplicity reactivation by the T-even bacteriophages.

The recombination which is occurring has been demonstrated without resorting to the conventional genetic approach of examining "marker" transfer. Consequently, the complications encountered in such studies, such as heterozygosis and phenotypic mixing, are not a problem. The results indicate that reactivation and consequently recombination is a very efficient process. Whether such recombination occurs with similar frequency or efficiency between normal unirradiated particles in the one cell, or whether ultraviolet irradiation is stimulating recombination (Jacob and Wollman, 1955) is not known. Even so, for conventional genetic studies with influenza virus, the use of irradiated parents in the study of genetic interchange presents several new lines of approach. The frequency of recombination appears to be highly efficient and offers a means of breaking down linkage. Following small irradiation doses, the progeny produced by multiplicity reactivation is fully infectious and so a means of examining recombination in the absence of incomplete virus formation exists. The whole subject has considerable bearing on the nature of the replication and function of ribonucleic acid.

Returning again to the problem of incomplete virus formation, which was considered in the preceding section,
more information is now available concerning the conditions under which it occurs. The first is that regardless of strain, for a given irradiation dose, incomplete virus formation always occurs at a characteristic input. The second point is that as the radiation dose increases, the ID$_{50}$/HA ratio of the incomplete virus produced progressively falls.

The first point implies that the "incomplete gradient", which is observed when fully active virus of different strains produce incomplete virus, does not exist when the same strains produce incomplete virus under conditions of multiplicity reactivation. The existence of this "incomplete gradient" has been used as an argument against the multiplicity dependence of incomplete virus formation, particularly as the strain LEE does not form any incomplete virus on multiple infection (Fazekas de St. Groth and Graham, 1953, 1955). However, from Fig. 10 it is apparent that under conditions of multiplicity reactivation, strain LEE produces incomplete virus in exactly the same way as other virus strains. In the circumstances of multiplicity reactivation, virus particles are obliged to co-operate together to produce a complete copy, which then results in virus production. So it seems that as more units become available than are needed to produce a single copy, incomplete virus results. So that, not only is multiplicity a necessary requirement for incomplete virus formation, but
the effect seems to be produced through the co-operative interaction of virus genomes. This form of co-operation may be interpreted in terms of the recombination which characterizes multiplicity reactivation. Incomplete virus formation, in these circumstances, may depend on the formation of two complete sets of units. Alternatively, an excess of only a few of these units may be needed, in which case, the question arises as to whether it is any particular unit or units which are required to be in excess. Another possibility is that when a number of copies of individual genome sections are available to induce replication, competition for a limited number of replication sites occurs amongst these units. The outcome of this competition could be a faulty duplication characterized by over-production of some units, and absence or under-production of others. This is reflected in the make-up of deficient progeny particles which contain only partial replicas of parental genetic material. This is incomplete virus. The gradient in capacity to produce incomplete virus shown by normal virus strains then simply reflects the efficiency with which this unit substitution or competition can occur, and may be largely affected by synchrony.

The second feature of incomplete virus formation under conditions of multiplicity reactivation, namely increasing incompleteness with increasing radiation dose, then becomes the
result of increasing numbers of units competing for a limited number of replication sites, with progressive imbalance in the product produced.

While such interpretations are purely speculative they attempt to unite the obvious similarity between the process which leads to reactivation, and that which leads to incomplete virus formation. This similarity will be further considered later.
SUMMARY.

The inactivation of influenza virus by ultraviolet light is a one-hit process. The multiplication of virus preparations inactivated by ultraviolet light has been studied.

Multiple infection of cells with irradiated preparations leads to the production of virus. These preparations do not contain sufficient residual active virus to account for this yield. The amount and nature of the virus produced depends on the degree of irradiation and the number of irradiated particles per cell. The dose response curves obtained in these circumstances indicate that multiplicity reactivation is occurring.

The multiplicity reactivation found for influenza virus appears to be a very efficient process, and no strain difference between the strains MEL, BEL, and LEE in capacity to reactivate was detected. The predictions of the recombinational theory of multiplicity reactivation, proposed by Luria and Dulbecco (1949) for a unit number of six agree well with the observed multiplicity reactivation behaviour of influenza virus.

Under conditions of multiplicity reactivation, the incomplete virus gradient previously encountered disappears. The implications of this finding are discussed.
CHAPTER III.

THE NATURE OF INTERFERENCE.

INTRODUCTION.

In general, the term interference is used to describe all those circumstances in which the presence of one type of virus prevents the multiplication of a second in a normally susceptible host. However, with influenza virus the phenomenon has a more specific connotation. Inactivated influenza viruses are capable of preventing the multiplication in cells of subsequently entering, infective virus; but this blocking effect is a slowly established, intracellular effect. Cell receptor removal, which can be effected either by the virus enzyme or by RDE will prevent the multiplication of virus, but this particular effect is not generally regarded as interference. Both homologous and heterologous forms of inactive virus are capable of inducing interference.

Some studies of interference have been carried out under conditions where receptor destruction has dominated the effects encountered. Baluda (1957) has described "interference" phenomena for Newcastle disease virus which can be ascribed to this effect (Baluda, 1959), and the same explanation appears to hold for the "overcoming of interference" described by
Isaacs and Edney (1951a).

Influenza viruses inactivated in different ways differ considerably in their ability to induce interference (Henle, 1950; Isaacs and Edney, 1950a, 1951b; Fong, 1953). Virus inactivated by ultraviolet irradiation is generally regarded as being the best interfering agent. However, adequate information concerning the manner in which cells blocked with ultraviolet inactivated virus respond to challenge has not been obtained. On the other hand, detailed quantitative information is available concerning the establishment of interference and the response of interfered cells to challenge when heat inactivated virus, a relatively poor interfering agent, is used to establish interference (Fazekas de St. Groth et al., 1952; Fazekas de St. Groth and Edney, 1952). Whether the difference in efficiency of production of interference shown by heated and irradiated preparations is simply a matter of degree, or whether entirely different mechanisms are operating cannot be decided from the available evidence. Similarly, it is not possible to determine whether homologous and heterologous forms of interference operate by the same or different mechanisms. At the present moment, there is just not sufficient evidence available to answer these questions. Such limitations make the task of discussing the nature of influenza virus interference difficult. However, some of the current problems
associated with the nature of influenza virus interference will be briefly discussed.

The results of Fazekas de St. Groth and Edney (1952) suggest that the presence of a single heat inactivated virus particle per cell completely prevents the multiplication in that cell of subsequently entering, infective virus. A simple relation between the dose of interfering virus administered, and the fraction of cells blocked was obtained. Cells which did not receive an inactive virus particle remained completely susceptible. Cells which do receive one or more inactive virus particle achieve a state of interference which cannot be overcome, regardless of the size of the challenge dose.

There is here the clear suggestion that the inactive virus particle itself is responsible for the blocking effect produced. However, observations by Isaacs and co-workers (Isaacs and Lindenmann, 1957; Isaacs et al., 1957; Lindenmann et al., 1957; Burke and Isaacs, 1958a, 1958b; Isaacs et al., 1958) complicate the issue. These authors found that cells which have received inactive virus release a substance which in physical-chemical properties is quite distinct from the virus particle, and which is itself capable of inducing interference. This substance has been called interferon. In keeping with their relative capacities to produce interference, ultraviolet irradiated preparations induce much more interferon production.
than do equivalent amounts of heat inactivated virus. Isaacs and Burke (1959) have proposed an explanation of interference on the basis of interferon production. They postulate that inactive virus induces cells to produce interferon, which may resemble an intermediate product of normal virus synthesis. Such altered cells, when superinfected by active virus, synthesise more interferon instead of a normal virus intermediate, and this accounts for interference.

There are some puzzling features associated with these observations. In the experiments of Isaacs and Lindenmann (1957), the degree of interference established in host cells receiving heat inactivated virus shows a similar response to dose as is found in the experiments of Fazekas de St. Groth and Edney (1952). With small inputs of interfering agent, only partial interference is established. Yet in these circumstances, sufficient interferon has been released from these partly interfered systems to be able to induce extensive interference in fresh host cell systems (Isaacs and Lindenmann, 1957 - Table 4). The question arises as to why this obvious excess of interfering agent does not induce further interference in the host cell system from which it is derived. In other words, with so much interfering agent available, why is interference not complete? The results of Fazekas de St. Groth and Edney (1952) suggest that the degree of interference established depends
solely on the proportion of cells blocked. It seems likely, however, that the existence of a substance released from such blocked cells which is itself able to interfere would obscure this precise dose relation. Such a product is in fact produced, but it does not disturb this relation. The significance of this paradox is obscure, but it is reasonable to suppose that at least for the type of interference established by heat inactivated agent, interferon plays little part in its establishment, regardless of whether interferon production is the means by which interference operates.

These observations refer mainly to the interference induced by heat inactivated virus. As mentioned above, nothing is known of the manner in which ultraviolet inactivated virus establishes interference in cells, or the manner in which such cells respond to challenge, although it is known that interferon is produced in large amounts. Similarly, nothing is known of the interference induced by interferon, except that it does not result in the production of more interferon. If a variety of types of interference exist, what is the significance and role of interferon in each? At the moment it is impossible to decide whether interferon production is of the utmost significance or is only an incidental aspect of influenza virus interference.

There is obviously a pressing need for a systematic
investigation of influenza virus interference. Unfortunately, the work to be reported on this subject in this thesis is only of a preliminary nature. The technique used in previous chapters of this Section was modified to study the nature of heterologous interference, using ultraviolet irradiated virus as interfering agent. Unfortunately, it has not been possible to investigate all the problems posed above. However, despite certain quantitative difficulties in assessing interfering multiplicities, this technique should prove to be useful in the further exploration of interference phenomena. Some evidence is obtained of how at least one type of interference may operate.
EXPERIMENTAL.

The Multiplication of Active Virus in Interfered Cells.

Pieces of allantois on shell are able to support the multiplication of influenza virus even when challenged more than twenty hours after being cut from whole eggs. However, considerable variability in the amount of virus produced in response to a standard challenge occurs among individual bits once they are kept for more than six hours. Consequently, these pieces are only suitable for single cycle experiments so long as they are used within this time. The object of the experiments reported in this chapter was to determine in what way, if at all, virus multiplies in interfered cells. Preliminary attempts to use bits in interference experiments, using ultraviolet inactivated virus as interfering agent, indicated that about eight hours were required for the establishment of interference. Hence, bits could not be used for full interference experiments. A compromise was sought by administering interfering agent to whole eggs and, once interference was established, cutting up these eggs and using the bits obtained for single cycle experiments.

The type of interference investigated was heterologous interference, using ultraviolet irradiated preparations of the influenza B strain LEE as interfering agent, and the A
strain MEL as challenge virus. The stocks of LEE used were irradiated for 60 seconds in the manner described in Chapter 2, so that each virus particle received on the average more than twenty radiation hits. Very large doses of this material would be required for any multiplicity reactivation to occur.

To determine the amount of interfering virus needed to establish interference the following experiment was performed. Four groups of six whole eggs were inoculated with 10, 20, 30 and 40 agglutinating doses of irradiated LEE respectively. After eighteen hours, standard sized bits were cut from all eggs, as well as from a number of uninterfered eggs, and all pieces were challenged with a large dose of active virus. Table 1 indicates the average yields obtained twenty-four hours after challenge from each group. With 10 AD's of interfering virus per egg, the degree of interference established was only slight. However 20 to 30 AD's per egg of inactive virus produced a marked reduction in yield. Any larger dose of interfering virus resulted in complete interference.

Prior administration of interfering virus does not in any way affect the adsorption of subsequently inoculated active virus. As a check, twenty-four de-embryonated eggs which had each received 50 AD's of irradiated LEE twelve hours previously were challenged with 70 AD's of active virus per
YIELDS OBTAINED AFTER INTERFERENCE.

<table>
<thead>
<tr>
<th>Interference Dose</th>
<th>YIELD a</th>
<th>HA-log$_{2}$$^b$</th>
<th>Percentage of Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td></td>
<td>5.4</td>
<td>100</td>
</tr>
<tr>
<td>10 AD's</td>
<td></td>
<td>5.2</td>
<td>87</td>
</tr>
<tr>
<td>20 AD's</td>
<td></td>
<td>2.2</td>
<td>11</td>
</tr>
<tr>
<td>30 AD's</td>
<td></td>
<td>1.5</td>
<td>6</td>
</tr>
<tr>
<td>40 AD's</td>
<td></td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

(a) Challenge dose $10^{7.4}$ ID$_{50}$ per bit.

(b) Haemagglutinin titre per 0.25 ml. Each figure represents the average value obtained for twelve pieces, obtained from six eggs.
egg. After allowing two and one half hours for adsorption, and one hour for receptor removal by RDE, the percentage of active virus adsorbed was 56%, which corresponds to the uptake of active virus by normal cells (Table 1, Chapter I).

The estimation of inactive virus multiplicity per cell is complicated by the use of whole eggs. Occasional eggs receiving 20 to 30 AD's of interfering virus show no sign of interference. This effect could be due to abnormally large amounts of allantoic fluid inhibitor, or to abnormally large numbers of allantoic cells. Overall, there is a narrow range between no interference and complete interference. Despite the fact that most preparations of strain LEE have low ID$_{50}$/HA ratios, if it is assumed that under suitable conditions something close to the standard ID$_{50}$/HA ratio of $10^{6.3}$ holds, then a dose of 20-30 AD's of irradiated virus represents a multiplicity of about one inactive particle per cell in whole eggs. So, in what follows, it has been assumed that in eggs showing typical interference following a dose of 20 AD's of irradiated LEE, a multiplicity of about one particle per cell has been obtained.

On the basis of the foregoing, interference experiments were carried out using a dose of 20 AD's of inactive LEE per egg. Having allowed sufficient time for interference to become established (usually eighteen hours), these eggs were
cut into bits of standard size and challenged in the usual way with two-fold dilution steps of active MEL. Control single cycle growth experiments in uninterfered eggs were performed at the same time, to standardize the challenge multiplicity estimates. Table 2 and Figures 1 and 2 show the typical multiplication behaviour of active virus in two normal and two interfered eggs. The results for control egg No. 1 and interfered egg No. 1 are plotted in Figure 1, while the results for the remaining pair are shown in Figure 2.

The setting for the scales of haemagglutinin and infectivity were obtained from the ID_{50}/HA ratios of the control eggs, which in each case were in the vicinity of 10^{5.2}.

If only those cells which have been untouched by interference are capable of yielding, then the response curve would be a shrunken version of the control. Obviously, this is not the case. Both the slope and position of the response curves obtained under these conditions of interference suggest that a co-operative form of multiplication is occurring. In fact, small interfering doses of about one particle per cell are capable of preventing the multiplication of single, active particles. However, when the multiplicity of active virus per cell is increased, a point is reached at which multiplication can be induced in some of the cells. So, in certain circumstances, interference can be overcome by increasing multi-
TABLE 2.

THE RESPONSE OF NORMAL AND INTERFERED CELLS TO CHALLENGE WITH ACTIVE VIRUS.

<table>
<thead>
<tr>
<th>CONTROL</th>
<th>Input Virus a</th>
<th>Egg 1</th>
<th>Egg 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution of Stock</td>
<td>ID&lt;sub&gt;50&lt;/sub&gt;/bit&lt;sup&gt;b&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt;)</td>
<td>Multi-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>HA-log&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1/20</td>
<td>7.18</td>
<td>16</td>
<td>4.4</td>
</tr>
<tr>
<td>1/40</td>
<td>6.88</td>
<td>8</td>
<td>4.2</td>
</tr>
<tr>
<td>1/80</td>
<td>6.58</td>
<td>4</td>
<td>4.2</td>
</tr>
<tr>
<td>1/160</td>
<td>6.28</td>
<td>2</td>
<td>4.1</td>
</tr>
<tr>
<td>1/320</td>
<td>5.98</td>
<td>1</td>
<td>3.3</td>
</tr>
<tr>
<td>1/640</td>
<td>5.68</td>
<td>0.5</td>
<td>2.7</td>
</tr>
<tr>
<td>1/1280</td>
<td>5.38</td>
<td>0.25</td>
<td>1.7</td>
</tr>
<tr>
<td>1/2560</td>
<td>5.08</td>
<td>0.13</td>
<td>0.7</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>INTERFERED</th>
<th>Input Virus a</th>
<th>Egg 1</th>
<th>Egg 2</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dilution of Stock</td>
<td>ID&lt;sub&gt;50&lt;/sub&gt;/bit&lt;sup&gt;b&lt;/sup&gt; (log&lt;sub&gt;10&lt;/sub&gt;)</td>
<td>Multi-&lt;sup&gt;c&lt;/sup&gt;</td>
<td>HA-log&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>1/4</td>
<td>7.88</td>
<td>89.6</td>
<td>2.7</td>
</tr>
<tr>
<td>1/8</td>
<td>7.58</td>
<td>44.8</td>
<td>1.9</td>
</tr>
<tr>
<td>1/16</td>
<td>7.28</td>
<td>22.4</td>
<td>1.5</td>
</tr>
<tr>
<td>1/32</td>
<td>6.98</td>
<td>11.2</td>
<td>0.7</td>
</tr>
<tr>
<td>1/64</td>
<td>6.68</td>
<td>5.6</td>
<td>-</td>
</tr>
<tr>
<td>1/128</td>
<td>6.38</td>
<td>2.8</td>
<td>-</td>
</tr>
<tr>
<td>1/256</td>
<td>6.08</td>
<td>1.4</td>
<td>-</td>
</tr>
<tr>
<td>1/512</td>
<td>5.78</td>
<td>0.7</td>
<td>-</td>
</tr>
<tr>
<td>1/1024</td>
<td>5.48</td>
<td>0.4</td>
<td>-</td>
</tr>
</tbody>
</table>

(a) Stock Virus titre 10<sup>8.10</sup> ID<sub>50</sub>/0.25 ml.
(b) Contained in a volume of 0.6 ml.
(c) Estimated multiplicity, using a value of 10<sup>5.5</sup> as the number of cells per bit, and subtracting 0.45 log<sub>10</sub> from the input titre per bit to allow for adsorption, and conversion of ID<sub>50</sub> to infectious units.
(d) Both haemagglutinin and infectivity titres per 0.25 ml.
**Figure 1.** The multiplication of active virus in interfered cells. Results obtained for Egg No. 1, Table 2. Open circles represent infectivity yields.
plicity. On the other hand, similar experiments with slightly
greater doses of interfering agent (40 AD's of LEE or more)
indicate that once the multiplicity of interfering agent per
cell is increased, this effect can no longer be demonstrated.
Interference in these circumstances cannot be overcome,
regardless of the input multiplicity of challenge virus.

A distinctive feature of this co-operative multi-
plication is the fact that under these necessarily high input
multiplicity conditions, the type of virus produced always
appears to be fully complete. This is quite distinct from what
is found with the co-operative multiplication which charact-
erizes multiplicity reactivation. However, just as with multi-
plicity reactivation, the maximum yields obtained never reach
the same values as obtained from normal cells, but are invariably
two to four-fold lower.

The Multiplication of Irradiated Virus in Interfered Cells.

Both multiplicity reactivation and the overcoming
of interference are co-operative processes. The possibility
immediately springs to mind that they may be aspects of the
same process. The manifestations of multiplicity reactivation
have already been explained in terms of a recombination. In
the case of interference some type of recombination could also
be occurring. The presence within cells of a number of
irradiation damaged units introduced by the interfering virus
particle may in some way provide the barrier which can only be overcome by a recombinational process. If multiplicity reactivation and interference represent the same type of process, then the operation of this barrier which diminishes the efficiency of active virus multiplication might also be expected to diminish the efficiency of multiplicity reactivation. On the other hand, if there is no diminution in reactivation efficiency of irradiated virus in this system, the two types of recombination may involve different things. In this case there would be no relation between the two co-operative processes.

Hence, the capacity of irradiated virus to reactivate in interfered bits which had received 20 AD's of LEE was determined. An 8.5 hit preparation of strain MEL was used as challenge, and the result of a typical experiment is shown in Figure 3. The position for the response curve of this irradiated preparation in normal cells is shown by the broken line. Obviously, multiplicity reactivation can occur with less efficiency in interfered cells than it does in a normal cell. A remarkable feature of multiplicity reactivation in these circumstances however, is that once again only complete virus appears.

These findings are suggestive but by no means conclusive evidence in favour of a recombinational process being involved in the overcoming of interference, which further
suggests that it is the virus genome itself in this case which is responsible for the establishment and maintenance of interference.
Figure 3. Multiplicity reactivation in interfered cells.
The multiplication of an 8.5 hit MEL preparation in an interfered egg. The broken line indicates the expected position of the response curve for such a preparation in non-interfered cells.
DISCUSSION.

The most significant finding which emerges from these studies is that a type of interference exists which can be overcome by the co-operative interaction of virus particles. The interference established by small interfering doses can be overcome in this way, while larger amounts of interfering virus establish a permanent interference. Any explanation of this phenomenon must not only account for the narrowness of the interfering virus dose range in which co-operation can occur, but also for the fact that the progeny of such co-operation are invariably complete virus. The suggestion has been advanced that this co-operation represents some form of recombination. At this stage, it is difficult to conceive any other suitable explanation for such a process, and so the following tentative interpretation is proposed.

The capacity of influenza virus to undergo multiplicity reactivation has been explained in terms of the existence of a number of independent virus subunits. Such sub-units may be involved in the overcoming of interference. However, the number of virus particles required to overcome interference provides many copies of these individual subunits, and yet the response curves obtained suggest that only one complete set of units is being attained. This phenomenon indicates that some factor is acting which greatly reduces
the chance of obtaining a complete set of units, despite the large numbers available. The irradiation damaged sub-units of the inactive virus particle used to establish interference, or some derivatives of these, could constitute this factor. Hence, the recombinational process could be between active units provided by the challenge virus and altered units derived from the interfering agent. Even with small interference doses however, of about one inactive particle per cell, the presence of a great number of active virus units is just sufficient to provide a single copy of active virus, and so complete progeny are produced. When the interfering dose is only slightly increased, this recombinational process becomes insufficient for even a single copy to be produced, regardless of the multiplicity of challenge virus.

If single inactive virus particles were capable of inducing this relative type of interference in cells, while more than one particle per cell produced absolute interference, the amount of co-operation obtained should be proportional to the fraction of singly blocked cells for any interference multiplicity. The narrowness of the range in which co-operation does occur however, suggests that this is not the case. The narrow range may not reflect multiplication in cells which contain single inactive virus particles, but multiplication in cells which did not receive an inactive particle and have
been interfered secondarily. This secondary interference could be due to interferon. Ultraviolet irradiated virus preparations are known to produce large amounts of interferon (Lindenmann et al., 1957). If interferon production is actually the stimulation of cells by inactive virus to produce some form of unit replica, then the identity of interferon is established. Burke and Isaacs (1958b) have shown that interferon is released late in the course of normal virus multiplication, and so it may even represent normal virus sub-units. However, the uptake by uninterfered cells of large numbers of these units may induce a state of relative interference, which requires co-operation by active virus units to be overcome.

This explanation implies that, just as in the case of heat inactivated virus, ultraviolet irradiated particles are able to induce a state of interference in cells which cannot be overcome. Unlike heat inactivated virus however, they produce sufficient interferon to be able to establish a secondary form of interference, which can be overcome by co-operation. This in part contributes to the greater efficiency of irradiated virus as an interfering agent. These studies not only imply a possible role in interference phenomena for interferon, but suggest that it is a form of virus genome sub-unit. Interference itself is then, as suggested by Isaacs and Burke (1959), a modified form of reproduction induced by the
inactivated virus particle.
SUMMARY.

The nature of heterologous interference, using ultraviolet irradiated interfering agent has been investigated. The capacity of this agent to establish interference appears to be a very efficient process. Small doses of inactive virus, of the order of one virus particle per cell, establish a state of interference which can be overcome by the co-operative interaction of active virus. Irradiated preparations capable of multiplicity reactivation are also able to overcome this type of interference. The progeny produced as a result of this co-operative multiplication is invariably fully complete. Larger doses of interfering agent establish a solid interference which cannot be overcome, regardless of the size of the challenge.

An interpretation of these observations and of the role of interferon is presented.
Three aspects of influenza virus multiplication have been considered in this section of this thesis. In the first place, the multiplication of active virus in susceptible cells was examined. Single, active influenza virus particles are capable of initiating infection. However, when two or more active virus particles participate in the production of virus, a product appears which differs from that produced by single virus particles. The notion has been developed that this characteristic product, incomplete virus, is the result of an interaction between parental particles.

Secondly, the multiplication of virus inactivated by ultraviolet irradiation was examined. Provided the radiation dose is not large however, the presence of a number of irradiated virus particles in a cell will result in that cell yielding, although individually each virus particle is quite incapable of multiplying. This multiplicity reactivation provides another example of virus interaction. A collaboration between particles is essential for virus to be produced.

Finally, the nature of interference was examined.
Normally, active virus cannot induce multiplication in interfered cells. With some doses of interference however, a large input of active virus will succeed in producing yield. This success is again the result of virus interaction.

The one thing in common shared by the processes which lead to incomplete virus formation, to multiplicity reactivation, and to the overcoming of interference is that each process is the result of interaction between virus particles.

The ability of irradiated virus to reactivate, or of active virus to overcome interference shows that in certain circumstances this interaction between virus particles is essential for multiplication to occur. On the other hand, the capacity of fully active virus to produce a distinct product if there is interaction, namely incomplete virus, suggests that influenza virus does not undergo interaction only when the circumstances demand it, but that this interaction is a constant feature of multiple infection. Consequently, it is possible to regard this capacity to undergo interaction as a characteristic feature of influenza virus multiplication.

If this is so, it is now possible to draw a distinction between the multiplication induced by single virus particles, and particles working together to produce an effect.
The nature of the various interactions considered in this section remain for the most part obscure, and so, the term co-operation, implying a working together to produce an effect, has been used to describe these circumstances. The two forms of multiplication which may be distinguished can now be called simple and co-operative forms, respectively. The multiplication of both single active and single incomplete virus particles provide examples of simple multiplication.

It now remains to define co-operation. Co-operation may be considered to be any effect or process which requires the participation of two or more virus particles within the limits of a single cell. This interaction may be effected in many ways. It would result from a direct interaction between the virus particles themselves, or could be mediated through some agent or product. Direct collaboration could for example operate by the direct interaction or interchange of genetic materials, or it may require the accumulation or interaction of non-genetic yet functionally important elements. Yet again, it may represent a necessary interaction between genetic and non-genetic elements. On the other hand, co-operative mechanisms could be conceived in which the collaboration is mediated indirectly by derivatives of virus constituents.

Only in the case of multiplicity reactivation is there sufficient evidence to suggest how co-operation occurs.
In the case of interference and incomplete virus formation, the mechanisms operating remain obscure, and much work is required to clarify the position. However, despite the fact that the idea of co-operation covers many possibilities, it offers an interpretation of several hitherto unrelated features of influenza virus multiplication. It is an hypothesis which ascribes to three diverse phenomena a common fundamental mechanism, and so may provide a basis for the clearer understanding of the biology of influenza virus.

Most well studied viruses provide instances of co-operative processes. Virus recombination is an obvious example. The concept of co-operation here developed may have application as a general descriptive term for all occasions when the concerted action of virus particles is required to produce an effect. In this way, co-operation may prove to be of general biological significance.
Diluents

Saline: 0.85% sodium chloride in distilled water.

Citrate Saline: 0.45% sodium chloride, 2.0% sodium citrate, 0.0063% citric acid, dissolved in distilled water and adjusted to pH 7.2 with 0.025 M Tris buffer.

Calcium Magnesium Saline: 0.85% sodium chloride, 0.0079% magnesium chloride, 0.0028% calcium chloride, dissolved in distilled water, adjusted to pH 7.2 with 0.02 M borate buffer.

Gelatin Saline: 0.5% gelatin in Ca Mg saline.

Standard Medium (SM): 0.8% sodium chloride, 0.06% potassium chloride, 0.08% calcium chloride, 0.005% magnesium chloride, 0.03% glucose, 0.2% gelatin, 0.01% chloramphenicol, 0.00025% Phenol red, adjusted to pH 7.0 with normal sodium hydroxide.

Red Blood Cells

Fowls were bled from the wing vein into citrate saline. The cells were centrifuged and washed three times in saline. After the final centrifugation, the packed cells were stored at 4°C. Red cell suspensions of 5% strength were prepared when required. No packed cells older than 3 days were used.

Titration of Haemagglutinin

Volumes of 0.25 ml of diluent were delivered from an automatic pipetting machine into each cup of transparent plastic trays. Normally, saline was used as diluent. Serial twofold dilutions of virus were prepared, using a Takátsy (1955) spiral loop. A standard drop (0.025 ml) of 5% fowl red cells was then added, the trays shaken and allowed to stand at room temperature. The pattern
of settled cells was read half an hour later. Partial agglutination was taken as endpoint.

The error of a single titration is about 8 per cent (Fazekas de St. Groth and Graham, 1955). When even greater accuracy was required, red cells were added as 0.25 ml. volumes of a 1% suspension from an automatic pipetting machine.

When haemagglutination tests titrations were carried out in the presence of RDE, trays were chilled to 2°C. before the addition of cells.

Receptor Destroying Enzyme

Several batches of high titre RDE were kindly provided by Mr. G.L. Ada of the Walter and Eliza Hall Institute, Melbourne. When titrated by the method of Burnet and Stone (1947) this RDE had a titre of 10,000 units per ml.

Titration of Enzyme

Ovomucin. About 400 ml. of egg white was added to 1500 ml. of distilled water at 2°C. The precipitate which results was centrifuged down and washed three times with 0.5% Na Cl solution. The washed precipitate was dispersed in a minimal volume (about 70 ml.) of 10% Na Cl, and then reprecipitated by addition of 1200 ml. of distilled water. This new precipitate was deposited by centrifugation and again dispersed in 10% Na Cl in distilled water, providing about 100 ml. of ovomucin. This material served as substrate for enzyme titrations.

Ovomucin Titration. Serial two-fold dilutions of inhibitor
in 0.25 ml. of saline were prepared in plastic trays. To each cup was added one drop of IEE allantoic fluid, previously heated at 56°C. for 30 min. and diluted so as to contain five agglutinating doses of virus per drop. After shaking, the mixtures were held for 30 min. at room temperature, when 0.25 ml. of 1% fowl red cells was added. The pattern of settled cells was read one hour later. Partial agglutination was taken as end-point, and the reciprocal of the inhibitor dilution showing the endpoint represents the inhibitory titre. A series of such titrations against red cells from a number of different birds were carried out to determine which birds would provide a suitable source of "sensitive" fowl red cells (Anderson, 1948).

**Virus Enzyme Titration.** Serial two-fold dilutions of virus were prepared in 0.25 ml. volumes of saline, and to each was added five inhibitory units of ovomucin. After incubation for one hour at 37°C, the trays were placed in a waterbath at 65°C. for 30 minutes to destroy the virus haemagglutinin, and then cooled to room temperature. Five agglutinating doses of indicator virus, in the form of strain IEE heated at 56°C. for 30 minutes, were added to each cup, and thirty minutes later 0.25 ml. of a 1% suspension of inhibitor sensitive red cells. The pattern of settled cells was read one hour later. Partial agglutination was taken as the endpoint, and the reciprocal of the virus dilution showing this endpoint represents the virus enzyme titre.
Electron Microscopy

In preparing samples for electron microscopy, the agar filtration method of Kellenberger and Arber (1957) was adopted. Collodion films formed over agar serve as a filter through which liquids and salts in the suspension to be investigated will diffuse into the agar gel. After this filtration, the film is fixed and floated off the agar onto specimen grids.

Preparation of agar plates. Agar plates consisting of 20 ml. volumes of 2% Difco Bacto-Agar in distilled water were poured into Petrie dishes 9 cm. in diameter. After solidification, the plates were dried in a 60°C. oven for one hour, during which time considerable water was lost by evaporation, and the adsorptive capacity of the plates thus increased.

Collodion films. A 0.32% solution of collodion in amyl acetate was poured over the surface of the dried agar plates, using 0.5 ml. per plate. Excess collodion was drained off, and the plates were inverted over filter paper for four hours.

Filtration. 0.2 ml. volumes of the preparation to be examined, usually consisting of equal parts of virus material and a standardized polystyrene latex suspension were carefully spread over the surface of the collodion film with a glass spreader. Once spread, the Petrie dishes were covered to avoid evaporation. When dry, the spread area was often difficult to identify, so its extent was marked out with a grease pencil on the bottom of the dish. Filtration occurred quite rapidly, and the preparations were then fixed by inverting the plate over a small amount of osmic acid.
Microscopy. After fixation, that portion of the collodion membrane covered by the dried preparation was cut into small pieces, of about 5 sq. mm. in size and floated off the agar onto copper electron microscope grids. These preparations were then shadowed with uranium, and photographed in an RCA EMU-3B electron microscope.

Latex. Several latex suspensions were used in these studies. For the particle counts reported in Section I, Table 1 (Page 24) a latex suspension of approx. particle diameter 1100 m$m\mu$, and of concentration $6.88 \times 10^9$ particles per ml. was employed. For determining the density distribution of virus particles, as recorded in Section I, Table 3 (Page 44), the latex was 365 m$m\mu$ in diameter, and used at a concentration of $2.25 \times 10^{10}$ particles per ml. A clumping together of latex particles was frequently encountered, but this could be overcome by sonicating the mixture for about one minute with a Mullard 50 watt ultra-sonic drill, E.7680.

Eggs

Fertile eggs were incubated at 38°C. and 55-65% relative humidity, and were mechanically turned twice a day. They were candled for use after 11 days incubation.

Virus

Strains. The following egg adapted strains of influenza virus were used.

PR8 (Francis, 1934)
MEL (Burnet, 1935)
BEL (Burnet and Bull, 1943)
LEE (Francis, 1940).
Preparation of Stocks. To obtain stocks of fully active, standard virus, eleven-day eggs were inoculated allantoically with $10^{2-10^4} \text{LD}_{50}$ of virus, contained in 0.05 ml. volumes of gelatin saline or SM. The eggs were then incubated at 36°C for 24 to 30 hours, depending on the virus strain, at which time they were chilled and harvested individually. Routinely, these stocks were kept as individual allantoic fluid preparations.

To obtain incomplete virus preparations, serial passage of undiluted virus was initiated, as described by von Magnus (1951b), commencing with standard virus of high titre. Usually, 0.2 ml. of undiluted allantoic fluid were used as inoculum, and eggs were incubated for 24 hours before harvesting.

Storage. Allantoic fluid containing virus was dispersed in 0.5 ml. volumes to 1 ml. glass ampoules, snap frozen in dry ice-alcohol mixtures, and stored in either a dry ice cabinet or a Revco unit, Model SZR-653 operating at -72°C.

Concentration. For density gradient centrifugation, concentrated stocks of virus were prepared by sedimentation in the ultracentrifuge and resuspension. Freshly harvested, infected allantoic fluid, usually about 500 to 600 ml. but distributed in 90 ml. volumes, was subjected to centrifugation for one hour in a Spinco, Model L preparative ultracentrifuge, using head 21 and operating at 16,000 r.p.m. (17,000 g). The deposits were suspended in saline and pooled, so that a concentration of approx. one hundredfold was achieved. Examination of such preparations in the
electron microscope however revealed that extensive aggregation occurred under these conditions. Sonication of such concentrated stocks for one minute with a Mullard 50 watt ultrasonic drill, E7680 produced a state of dispersion indistinguishable from that found in allantoic fluid preparations.

**Titration of Infectivity**

Infectivity titrations were carried out by the method of Fazekas de St. Groth and White (1958), using pieces of surviving allantois in plastic trays. The method is as follows.

Each cup of clean plastic trays received 0.3 ml. volumes of SM. Eleven-day old eggs were de-embryonated, rinsed twice with SM, and cut into 6 x 6 mm. squares of shell with adherent membrane. One piece of tissue-on-shell was placed in each cup of the prepared trays.

Routinely, eight two-fold dilution steps of the preparation to be titrated were prepared, and 0.025 ml. of each dilution was added to each of ten egg pieces. In this way, one tray served for a complete infectivity titration.

Once inoculated, the trays were stacked in groups of five, surrounded by a strip of moistened lint, and wrapped in a polythene sheet. They were then placed on a horizontal shaking machine working in a warm room at 35°C, and incubated for 60 hours.

At the end of this period, the trays were dismantled, the egg pieces removed and one drop of 10% fowl red cells was added to each cup. Positives were indicated by the presence of haemagglutination in the cup. Endpoints were determined according to the method of Reed and Muench (1938).
De-embryonated Egg Technique

Eggs were de-embryonated by cutting off the pointed, albumen end of the egg and tipping out the contents. In this way, only the membrane lined shell remains. Eggs incubated until 14 or 15 days are most suitable. The emptied eggs were thoroughly rinsed with SM, and sealed with aluminium milk bottle caps. The eggs were placed in wooden holders capable of holding up to 36 eggs. The holders were placed on a rotating machine, operating in a warm room at 35°C, which rolled the eggs through 360° twelve times per minute with their long axes inclined at 17° to the horizontal. Eggs were normally given an hour or so to become stabilized at 35°C. before receiving the inoculum through a small hole in the cap.

The Single Cycle Yield Experiment

Pieces of shell 1 sq.cm. in area with allantois attached cut from eleven day old chick embryos were suitable for this type of experiment. These were prepared by marking off the surface of the egg into measured 1 sq.cm. areas, de-embryonating the egg carefully from the pointed end, and cutting with scissors, avoiding any areas which contain remnants of mesentry. With practice, bits could be cut without measurement and not affect the reliability of the results. At least twenty such pieces could be obtained from a single egg, and this was sufficient for a complete experiment. The bits were then placed individually in cups of large Perspex trays, almost identical to those described in detail by Fulton and Armitage (1951). These trays contain one hundred cups arranged in ten rows of ten, each of capacity about two ml. A series of two-fold dilution steps of stock
virus in SM was prepared and 0.6 ml. of each was added to each of two egg bits in the trays. In practice, ten dilution steps were employed, using twenty egg pieces. All operations were carried out in a constant temperature room at 35°C, and it was important that trays and medium were adequately warmed before use.

After the addition of virus, Perspex lids were screwed onto the trays, and they were shaken on a horizontal shaking machine working in the warm room for one and a half hours, the period allowed for virus adsorption. Further adsorption was prevented, and any surface bound, unadsorbed virus was released by addition at this time of about 100 units of RDE, which was allowed to act for one hour. Two and a half hours after infection, the inocula were removed and kept for titration to determine the amount of adsorption. Bits were rinsed at this time with several changes of fresh medium. Finally, 0.7 ml. of medium was added to each bit, and the trays returned to the shaker for another five to five and a half hours. The removal of cell receptors by RDE allows instantaneous release of all newly formed virus into the medium (Cairns and Mason, 1953). At the conclusion of the experiment, the bits were removed and the yields titrated for haemagglutinin and infectious virus content.
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consolidation initiated by the viral inoculum.


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