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Structure of Influenza Virus

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This discussion is concerned with the topic which for the last 10 years has been the main preoccupation of my laboratory, the structure of influenza virus. This grew out of a more limited attempt to understand the significance of hemagglutination by the virus and has been greatly influenced by our recent work on the genetic behavior of influenza viruses. Most of the new work that I have to report is due to my colleague, G. L. Ada, whose investigations on the nucleic acids of influenza virus have, I believe, provided a very valuable chemical basis for the functional understanding of influenza virus structure.

At the present time, viruses occupy a key position in relation to general biological thought. As the smallest agents showing the essential characters of organisms, they provide virtually the only hope that their structure may be simple enough for comprehension in terms of accepted physicochemical concepts. I have some unorthodox doubts about the applicability of such concepts, even in principle, to the interpretation and control of essential biological phenomena, and I should prefer to regard viruses as providing the best field for the development of the complementary concepts that will eventually be needed.

Virology in a sense brings to a focus on a single set of phenomena the disciplines of organic and physical chemistry, on the one hand, and of genetics and cytology, on the other. In the background we have the humanly important implications of our subject for pathology and clinical medicine and for epidemiology and public health.

Influenza virus has its special human importance as potentially the greatest killer under modern conditions of all the pathogenic microorganisms. The 1918–19 pandemic set back the population growth of India by a decade. In the last analysis, an understanding of the structure of influenza virus must be incomplete if it does not provide, at least in principle, means by which pandemic influenza can be forecast and prevented. It is possible, however, to forget completely the medical importance of influenza and to study the virus in its interaction with the susceptible cells of the chick embryo, simply as a controllable laboratory phenomenon to be elucidated. This is the approach that has provided the data and ideas with which I am concerned in this article.

The general character of laboratory work in influenza virus is well known. For the time being, the chick embryo has not yet given place to tissue culture as the standard method for cultivation of the virus. A particle of influenza virus is recognized and in a sense defined by the fact that when it is introduced into the allantoic cavity of the chick embryo, a continuing sequence of infections of the lining cells is initiated which results finally in the liberation of very large amounts of descendant virus into the allantoic fluid. The presence of this virus is recognized in practice by the fact that the fluid has now acquired the capacity to agglutinate red cells. It might also be detected by examining suitable preparations of the fluid by electron microscopy. The virus may be present as spherical bodies about 100 millimicrons in diameter or in filaments of somewhat smaller diameter and of variable but sometimes relatively enormous length.

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Quantitative Work

All quantitative work on influenza virus is based on the use and intercomparison of the following three methods.

1) The infectivity—that is, the number of functionally active units—is determined by inoculation of appropriate dilutions into large enough numbers of chick embryos or their equivalents. The fact of infection is recognized by the hemagglutinating activity of the fluid.

2) The content of virus particles with functional surface qualities, irrespective of whether they are infective or not, can be estimated by suitable quantitative methods for estimating hemagglutinating power.

3) The content of morphologically typical particles can be estimated by electron microscopic methods. The presence and approximate number of filaments can be established by simple dark-field microscopy.

The application of such methods by Hirst, the Henles, Fazekas, Horsfall, and others (*1*) has shown the sequence of events in a susceptible cell. This sequence can be summarily stated as follows.

1) The virus particle attaches itself to the free surface of the cell, the union being mediated by an attachment of an enzyme-like component of the virus surface to prosthetic groupings (recently defined by Gottschalk, *2*) of cell-surface mucoprotein.

2) The virus particle enters the substance of the cell and rapidly loses its existence as an infective particle in the sense that an extract of that cell is no longer infective for a fresh embryo.

3) About 3 hours after infection, new virus is detectable in the cell; and from about 4 hours onward, new infective virus is being liberated from the cell. There is good evidence to suggest that the production of new infective virus takes place at the free surface of the cell.

It is clear that the problem of virus structure has a twofold character. On the one hand, we are concerned with the

structure of the infective virus particle as it can be isolated from a standard infected allantoic fluid. On the other hand, we have the processes taking place in the infected cell by which a new generation of infective particles is produced. We are concerned here with what is virtually a new functional organism, the virus-infected cell. There are even superficial resemblances between the process of fertilization of the mature ovum and of infection by virus of a susceptible cell. In each case, the cell is impelled to activities whose end result is determined or at least strongly influenced by the genetic character of the intruding agent. In each case, we are concerned basically with the interaction of two genetic systems in a single cell. It is possible to describe the infective virus particle as a relatively simple unit, but any description of the pseudo-organism, the infected cell, must necessarily be at least as complex as that of the cell itself.

Physical Structure and Chemical Composition

Most of the work has been done with the well-adapted standard strain of influenza virus A—PR8. In allantoic fluid virus, the particles are mainly spherical with only a few short filaments. The particles show an average diameter that varies according to the method used in preparing the specimen for electron micrography. With freeze-dried preparations, Williams (*3*) found that the average diameter is 82 millimicrons, and that in all preparations not subjected to sorting out processes, there is a considerable range of size. There is no clear indication of internal structure.

Although they are rare and inconspicuous in PR8 preparations, filaments are very characteristic of a number of recently isolated strains. In their most typical form, they appear in electron micrographs as uniform cylindrical objects approximately 70 millimicrons in diameter and of lengths which may be anything up to 20 or 30 microns. They very often show a "knob" of slightly greater diameter at one end of the filament.

In a later section, something will be said about the behavior of filaments, but here I wish merely to state the evidence for regarding them as "influenza virus." In the first place, they are well-defined morphological objects, which are seen only in fluids from embryos infected with the virus. They are readily adsorbed to red cells or red-cell ghosts and can be eluted either spontaneously or by the action of the receptor-destroying enzyme. Donald and Isaacs (*4*) found that a single filament has more activity as hemagglutinin than a single spherical particle and that sonic disintegration sharply increased the hemagglutinin titer of a preparation containing a high proportion of filaments. The results of infectivity titrations were indecisive, but they were consistent with the view that each filament was equivalent to approximately one infective unit. Infectivity, however, seems to be neither increased nor decreased by manipulations that break down the filamentous structure.

The chemical composition of purified virus particles from allantoic fluid has been reported on by various authors. For the most part, the results obtained are very similar to the analyses of cytoplasm from animal cells. There is present in terms of dry weight the following. (i) Lipids, 20 to 30 percent, including neutral fat, phospholipid, and cholesterol. (ii) Carbohydrates, approximately 5 percent, much of which is probably associated with mucoprotein, for it includes galactose, mannose, fucose, and hexosamine; ribose is present as a component of nucleic acid. (iii) Nucleic acid, 1 percent ± of ribosenucleic acid only. (iv) Protein, 60 to 70 percent, the amino acid composition of which is close to that of animal protein generally.

There is much to suggest from these analyses that a considerable proportion of the material contained in what is conventionally regarded as purified virus particles is very directly derived from the host cell. Further evidence in this direction is given by Knight's well known finding of components with the immunological specificity of host tissue in washed influenza virus (*5*).

As in so many analogous situations, the identification of material as specifically viral depends on functional rather than analytic, chemical tests. Of the functional components of the particle, we can recognize two by serological methods usually referred to as viral antigen and soluble complement-fixing antigen and one, presumably carried by the same complex responsible for the viral antigen, by its specific adsorption to and/or enzymatic action on red cells or equivalent mucoprotein.

The relationship of these functional components to the morphological and chemical components has not been satisfactorily worked out for influenza virus. However, practically all the published findings, as well as current work by Ada (*6*), suggests that the situation is very similar to or identical with what has been found for fowl plague virus by Zillig and his collaborators (*7*). Using repeated ether treatment as a means of disrupting the virus particle, they found that a hemagglutinin unit considerably smaller than the virus could be obtained. This material has recently been reported as being composed of protein with, probably, some contaminating polysaccharide but no nucleic acid. It is reasonable to believe that its specific attributes are based on the protein. The second functional component from disrupted fowl plague virus is the soluble complement-fixing antigen which can be shown in electron micrographs as small elongate granules. Ada has recently found that similar soluble complement-fixing antigen can be obtained from purified influenza virus similarly treated with ether. Since the complement-fixing activity is not shown by the intact particles, it must be assumed that the specific pattern involved is not at the virus surface.

It may be mentioned here that the action of ether on influenza virus is apparently greatly modified by minor environmental circumstances. With freshly redistilled ether we have failed to confirm the statement that exposure of allantoic fluid virus to 20-percent ether at refrigerator temperature overnight destroys its infectivity. Prolonged and repeated treatment is necessary for disruption of infective particles.

Filamentous forms of recently isolated strains of influenza virus have in recent years been extensively studied at Mill Hill and in my own laboratory. We have made use of the fact that filaments can be seen and counted using simple dark-ground microscopy. This allows the quantitative study of agents capable of breaking down filaments. Our most interesting finding was that, with one partial exception, all the agents tested that were capable of hemolyzing fowl erythrocytes destroyed influenza virus filaments and that the end point for both types of lytic action was almost precisely the same. The test included such varied agents as hypotonic solutions, ether and chloroform, sodium lauryl sulfate and cetyltrimethylammonium bromide, *Clostridium welchii* α-lecithinase, cobra venom, and lysocithin. The exception, saponin, gave rise to distorted and beaded forms in dilutions corresponding to its hemolytic level, but the forms remained recognizable as filaments throughout. Disruption of filaments by ether or chloroform gave rise to a variable increase in hemagglutinin titer in fluids containing a high proportion of filaments but gave no change in infectivity. This parallels the finding of Donald and Isaacs with sonic disintegration.

Our conclusion is that influenza virus filaments may contain infectious units, probably in the knobs visible in electron micrographs, but as such they are noninfective. Their surface, however, includes virus specific antigen and enzyme. It resembles the surface of a red cell and by implication that of more typical avian cells in being very sensitive to disintegration by ether and other hemolytic agents. The spherical virus particle has been adequately identified with both the infective agent and the hemagglutinin. It is much

more stable than the filament, but its high content of lipid and the presence of mucoprotein suggest that it too has important affinities to the surface structure of vertebrate cells.

Mechanism of Fabrication

The relationship between spherical infective particle and virus filament can be understood clearly only in terms of the process by which the particle and filament are produced. This leads us to the consideration of the intracellular phase which I have already described as the interaction of two genetic systems to produce what is virtually a new organism—the infected cell.

This interpretation is based essentially on three experimental findings: (i) the disappearance of infectivity after infection is initiated, (ii) the occurrence of a variety of genetic interactions between virus types when appropriate double infections are induced, and (iii) the presence of host-specific components in the virus progeny.

Any detailed understanding of what is taking place must be built up in rather speculative fashion from disconnected fragments of information against the background of what is known about normal synthetic processes in the cell. The relevant information may be divided into four classifications: (i) that concerned with the behavior of ribose nucleic acid; (ii) the appearance and distribution of soluble complement-fixing antigen; (iii) the genetic evidence for the existence of a "replicating pool" of virus components; and (iv) evidence for the direct part played by the cell surface in assembling the virus.

Ada and Perry (*8*) have shown that the nucleic acid content of purified influenza virus is low—± 1 percent—and that only ribose nucleic acid is present. This has been confirmed in other laboratories by Hirst (*1*), Hoyle (*9*), and Liu *et al.* (*10*). The nucleic acid bases adenine, guanine, cytosine, and uracil are present in a ratio which differs significantly as between A and B strains and is sharply distinct from that of ribose nucleic acid isolated from normal allantoic membrane cells. Ada therefore believes that the pattern of bases in ribose nucleic acid isolated under various circumstances associated with influenza virus infection may throw important light on the processes involved. This is, of course, in line with the insistent evidence from various sources that synthesis of protein in the cell cytoplasm is intimately associated with ribose nucleic acid activity.

Ada's other findings may be summarized as showing (i) that soluble complement-fixing antigen extractable from infected embryo lung contains ribose nucleic acid that has a base constitution close to that from a similar fraction of normal cells; (ii) that virus produced in chick embryo lung and purified by standard processes has a similar content of ribose nucleic acid, but its base constitution deviates from that of allantoic virus toward the host cytoplasm pattern; (iii) that soluble complement-fixing antigen extracted from allantoic fluid virus has the characteristic base composition of virus ribose nucleic acid.

Interpretation of such results may have to wait on the better understanding of normal protein synthesis, but one point is clear. The mechanism by which virus patterns are conveyed to descendant virus requires ribose nucleic acid only—it is a genetic system in which desoxyribose nucleic acid is not directly involved.

The concept of a pool of replicating components is well established in the literature of bacterial viruses and is clearly also applicable to the cell infected with influenza virus. The genetic evidence is decisive on this point. Recombination of characters requires interpenetration of genetic material from the two parent forms. The existence of phenotypic mixtures—doubly neutralized hemagglutinin—in which the virus carries a mosaic of surface patterns shows further that not only the genetic but also the somatic components are extracted from a common pool of components. The appearance early in the process of soluble complement-fixing antigen with viral specificity offers a hint that this is an important component of the replicating pool.

In rather general terms, we can picture the replicating pool as being built up by the partial or complete taking over of a protein- and ribose nucleic acid-synthetic mechanism by virus components of which the most important may be the specific ribose nucleic acid of the virus particle. This results in the production, presumably by processes of direct or indirect replication, of (i) those host components needed for the metabolic support of the replicating process, (ii) genetic components of the virus, and (iii) somatic virus components of which the only demonstrable example is the surface component which appears to be responsible for hemagglutinin enzyme and viral antigen.

All the evidence obtainable from studies of allantoic multiplication of influenza virus and the related fowl plague virus points to the cell surface as the site of fabrication of the virus particles and filaments. Cairns and Mason by comparing the rise of virus titer in allantoic membrane extracts with that free in the allantoic fluid, both with and without treatment with receptor-destroying enzyme, concluded that detectable virus came into existence virtually at the same time that it was liberated from receptor-destroying-enzyme-treated cells (*11*). It was as if, by passing through the host cell membrane, it acquired specific character.

Electron micrographs of sections of infected allantoic cells show recognizable virus only at the free surface. In the pictures of fowl plague virus published by Hotz and Schafer (*12*), the particles seem to be budding off the cell surface. All workers agree that filaments form as extrusions from the cell surface, the shorter examples of which are difficult to distinguish from the microvilli to be seen at the free surface of uninfected cells. Harford's (1955) pictures of the process in the epithelial cells of the mouse lung show what appears to be an accumulation of virus particles in cytoplasmic inclusions, as well as distortions of the endoplasmic reticulum (*13*). The published figures do not seem to be inconsistent with the view that the virus particles are formed at a surface between the fluid of a vacuole and the cell substance, but the conditions are clearly not very closely analogous to those in the allantoic cells. Cells from infections of chick embryo lungs are typically vacuolated and in an early paper of mine it was suggested that these vacuoles might contain virus (*14*).

Although it is not relevant to the final stage of fabrication of virus particles, it should not be forgotten that there is evidence of nuclear involvement in the early stages of infection, and gross nuclear damage is a characteristic feature of a proportion of necrotic cells.

Out of this discussion there emerges a fairly clear though perhaps rather superficial interpretation of the structure of the influenza virus particle and the mechanism by which it is fabricated.

Penetration of Cell

Omitting for the time being any discussion of the filaments, we can commence with the penetration of a virus particle into the cell and the liberation in the cell substance of the essential genetic components of the virus. On the basis of Ada's findings, it is legitimate to consider these as being wholly or in part composed of ribose nucleic acid, and as making intimate association with ribose nucleic acid- and protein-synthetic mechanisms in the cell. This interaction may begin with some sort of "tooling-up" of templates in the nucleus, but the main production of new virus components must be in the cytoplasm. One pictures first a mobilization of synthetic activity represented histologically by enlargement of the nucleolus and intense pyronin staining of the cytoplasm, particularly in the perinuclear region. If Ada's results can be taken at their face value, two types of ribose nucleic acid may both be associated with serologically recognizable viral patterns that are characteristic of

cell extracts having the host cell distribution of bases while a virus-specific distribution is shown in what is finally incorporated in the infective particle. The replication of genetic material and the concomitant production of somatic units (assuming that these are distinct from the genome) result eventually in a high concentration of viral material of all types in the superficial cytoplasm of the allantoic cell.

The fabrication of the virus particle is best pictured as a process by which somatic units (of the virus) infiltrate the surface membrane of the cell to build up a stable membrane containing lipid and mucoprotein of host origin as well as the viral protein. This membrane finds its maximal stability when it is enclosing a sphere of about 100 millimicrons in diameter, and the simplest possible assumption is that in forming such a sphere the corresponding volume of cytoplasm with its contained components of the replicating pool is trapped inside.

It would be very much in accord with such a view for incompletely adapted virus strains to induce quite frequently a minor deviation of the process of space enclosure by a developing cell surface-virus protein membrane so that, instead of a spherical bud, an indefinitely extruding cylinder is produced. This would be an appropriate interpretation of filament formation. There is some evidence that filaments have fewer points of attachment to red cell receptors than spherical forms, and the filament surface would therefore be less altered from the host cell surface than that of the spherical particle. Its similar reaction to lytic agents would thus be understandable.

We reach, then, a picture of the standard virus particle as a rather loosely organized unit containing two types of macromolecule whose specific pattern is determined by the genetic character of the parent virus or viruses. On the surface, there is imbedded in a stable mosaic with lipid and mucoprotein components of cell origin a number of macromolecular units which carry, presumably on a protein basis, the specific patterns responsible for enzyme activity and adsorption to cell mucoprotein and for serological character of the viral antigen type. These patterns are characteristic of the virus strain and are genetically determined. We believe that they should be regarded as somatic in character and that from the point of view of survival of the virus their character determines the readiness with which infection can pass from one susceptible cell to another and initiate infection.

Within the particle, there is a variable number of genetic units, probably ribose nucleic acid-protein complexes. From genetic studies it is believed that these complexes in influenza A strains may show qualitative differences of types broadly similar to what is postulated for chromosomal behavior in higher forms. Lind and I have postulated two "linkage groups," each containing a number of loci subject to mutation. There is some evidence that the division into two linkage groups merely indicates that a single genetic complex breaks most readily at a certain point. The viable particle contains a number, which we have guessed as between five and 50, of such genetic complexes. It is entirely in accord with the findings in regard to incomplete virus and the phenomena of recombination, especially in the field which we have referred to as redistribution of virulence, that the genetic content of individual virus particles should vary in random fashion from one individual particle to another.

Conclusion

I should like to summarize our concept of the influenza virus particle as follows.

The virus particle is a loosely organized unit in which a surface membrane, which is derived largely from the host cell but which owes its specific qualities to a regular reinforcement with virus protein, encloses a variable number of genetic determinants which are almost certainly of ribose nucleic acid-protein structure and a proportion of adventitious material from the host cytoplasm.

It is not a rigidly organized structure like a bacterial virus or one of the smaller viruses such as poliomyelitis or tobacco mosaic virus. It would be in line with the present concept to look on polio virus as an organized aggregate of genetic determinants of standard size instead of, as it were, a bag containing a loose collection of genetic units.

There may well be some prejudice against accepting such a simple and somewhat indeterminate structure for influenza virus. However, to anyone who has worked extensively with influenza virus it is unthinkable that we are dealing with a highly standardized unit. In particular, we can mention (i) the variable diameter of spheres and the alternate filamentous morphology; (ii) the existence of varying degrees of incompleteness with evidence of morphological and chemical variation in relation to this; (iii) the existence of phenotypic mixtures in the progeny of mixed infections; and (iv) the redistribution of virulence when certain pairs of virulent and avirulent strains produce recombinants.

I have made no attempt in this paper to elaborate any of the genetic evidence on the basis of which this concept was first developed. This has been given in full elsewhere (*15*).

What remains to be done is to penetrate more deeply into the nature of the genetic determinants. Here I think we can look for developments from the correlation of ribose nucleic acid base analyses with genetic studies, and I am very hopeful that the current interest in the structure of polio virus will eventually allow a similar combined chemical and genetic attack on one of the smaller macromolecular viruses.

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A mathematical investigation always obeys the law of the conservation of knowledge; we never get out more from it than we put in. The knowledge may be changed in form, it may be clearer and more exactly stated, but the total amount of the knowledge of nature given out by the investigation is the same as we started with.—HENRY A. ROWLAND.