VIRAL INTERFERENCE

Some Considerations of Basic Mechanisms and Their Potential Relationship to Host Resistance[1]

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I. INTRODUCTION

The profusion and diversity of the experimental data have led many students of the subject to consider that viral interference may be not one phenomenon, but many. This view stems in large measure from the inability to predict with confidence which virus will interfere with another in any particular type of cell. The result has been an imposing list of empirically determined pairs of interfering viruses which stands as eloquent testimonial to our ignorance of the mechanisms involved. The literature on viral interference has been collated and annotated so comprehensively by Schlesinger (1959) that it would serve no useful purpose to review his review. Inevitably, however, the present discussion will cover some of the same well-spaded ground.

This analysis of viral interference was approached with two specific objectives in mind: to examine the evidence for a unifying principle that may underlie the seemingly diverse reactions between viruses and cells that lead to interference; and to explore the possibility that these reactions may be implicated in host defenses distinct from specific immunity. These may be premature and quixotic hopes. A theory of the mechanism of viral interference must eventually

be based on chemical evidence, and needless to say, such evidence is scant. Nevertheless, the discovery of interferon (Isaacs and Lindenmann, 1957), a cell product of determinable chemical nature, holds out promise for future understanding of at least some of the cellular reactions involved in viral interference. The interpretations presented herein have been greatly influenced by this finding and are put forth with full awareness that they may be controversial.

An exact definition of viral interference is not possible at the present time. It has generally been assumed that the phenomenon represents competition between two viruses for the same host cell, but, as will be indicated later, the validity of this concept may be open to question. Nevertheless, meaningful interpretation of data still requires that the use of the term be restricted to events that take place at a cellular level. In this context, therefore, interference signifies acquired cellular resistance to viral infection. It would probably be wise to impose the additional qualification that inhibition of virus multiplication be the essential criterion of interference. Although interference may result in enhanced capacity of a host to survive infection or in decreased immunologic responsiveness, these are secondary manifestations of a cellular environment inimical to the infecting virus. By convention, the agent that induces the state of cellular resistance to infection is referred to as the interfering virus and

the one that is suppressed as the superinfecting virus. Under certain conditions, multiplication of both viruses may be inhibited. Although these designations imply dissimilarity between the virus pairs, it has long been recognized that related or even biologically indistinguishable viruses can interfere with each other. Contrary to custom, no attempt will be made to differentiate autointerference from heterologous interference between unrelated viruses. In the writer's opinion, classifications of interfering systems based on antigenic or other biological relationships among viruses may be unduly restrictive and may impose unwarranted complications in an analytical approach to the subject.

II. BACTERIOPHAGE "INTERFERENCE"

The term, viral interference, has not been popular among bacterial virologists (Adams, 1959). To these quantitative biologists, interference between animal viruses has borne the unhappy connotation of disconnected, and perhaps unrelated, events that transpire in dissimilar cells of a multicellular host. This view, of course, is true of almost all studies of mixed viral infections of intact animals. However, the advent of improved cell culture techniques has provided a means by which interference between two viruses that infect a single animal cell can be studied and compared with mixed infections of a bacterial cell. There are indications that the comparison will reveal more than a superficial resemblance. It seemed appropriate, therefore, to attempt a brief analysis of resistance of bacteria to superinfection with bacterial viruses in the hope of gaining some insight into possible mechanisms of interference between animal viruses.

There are probably three ways by which a bacterial virus can induce a state of resistance to superinfection with the same or different viruses. These three phenomena have been saddled with four rather unfortunate terms: resistance to lysis from without (Visconti, 1953); immunity of lysogenic bacteria (Lwoff, 1953); mutual exclusion, and the depressor effect (Delbrück, 1945). The latter two appear to be closely related and will be discussed together. Their similarity to interference between animal viruses has prompted the use of the convenient term interference in the discussion of mutual exclusion.

A. Resistance to Lysis from Without

Exposure of a bacterial cell to a high multiplicity of phage results in premature lysis of the cell without production of new phage progeny. This has been called lysis from without to distinguish the reaction from active viral infection (Delbrück, 1940). It resembles a toxic response in that virus inactivated by ultraviolet irradiation can produce the same lytic effect (Watson, 1950). Protection against lysis from without does not occur in bacteria previously infected with unrelated strains of bacteriophage (Doermann, 1948). However, Visconti (1953) found that bacteria infected with a low multiplicity of T2 phage rapidly became resistant to lysis from without caused by the related T2r strain of phage. The mechanism of this protective effect is not clear. It may possibly be a function of altered permeability of the host cell wall, which has been noted to occur after penetration by one or a few phage particles (Puck and Lee, 1955). If so, the challenge virus, or rather its deoxyribonucleic acid (DNA), may be prevented from entering the resistant cell in quantities sufficient to induce rapid lysis, or the lytic enzyme of the superinfecting phage may be blocked. This protective effect is reminiscent of acquired resistance to the toxic action of massive doses of influenza virus in animals pretreated with small inocula of biologically related viruses (Wagner, 1952). Neither of these protective effects sheds much light on the mechanism of viral interference and, in fact, both are probably unrelated to it.

B. Immunity of Lysogenic Bacteria

This phenomenon has been exhaustively studied by precise quantitative methods. Despite the presence of prophage in the lysogenic bacterium, certain unrelated virulent phages can adsorb on, penetrate, and multiply within the cell. The offspring of the superinfecting virus released from the lysogenic cell are indistinguishable from virus formed in nonlysogenic bacteria. If, however, the superinfecting phage is closely related genetically to the prophage carried by the lysogenic bacterium, it will adsorb and penetrate, but no multiplication will occur (Lwoff and Gutmann, 1950; Lwoff, 1953). Nor can a related temperate phage doubly lysogenize an "immune" bacterium, except under unusual circumstances (Bertani, 1956). One other exception to this generalization is a class of virulent phage mutants

capable of infecting bacteria lysogenized with related prophage (Bertani, 1953). A genetic basis for immunity to superinfection of certain lysogenic bacteria has been demonstrated by the elegant studies of Jacob and Wollman (1957). They showed that no cross immunity existed between 14 different temperate phages, each capable of lysogenizing *Escherichia coli* K12. Studies of bacterial recombinants by crossing experiments revealed that each prophage occupied a different genetic locus. It appears, therefore, that the DNA of a superinfecting temperate phage cannot be incorporated into the same site on the bacterial chromosome preempted by a related temperate prophage.

However, genetic competition may not be the only factor operative in immunity of lysogenic bacteria. Bertani (1956) found that a lysogenic bacterium resists superinfection with phage P2 if it carries prophage P2 at a secondary nonpreferred site on its chromosome. This led to the suggestion that "immunity is physiologically controlled (by some product of the prophage?) and not the result of competition for the standard site of prophage attachment." In addition to "homologous immunity," Lederberg (1957) has shown conclusively that *E. coli* or *Shigella dysenteriae* lysogenic for phages P2 or P1 also fail to support multiplication of certain heterologous bacteriophages. Growth of superinfecting phage is suppressed at some stage following attachment. It is difficult to invoke a genetic basis for this type of resistance to infection which, in many respects, resembles interference (mutual exclusion) between unrelated viruses. As one tentative hypothesis, Lederberg suggests that a specific inhibitor, possibly deoxyribonuclease (DNase), may be formed by the host cell in response to the superinfecting phage.

C. Mutual Exclusion and Depressor Effect

Approximately 8 to 10 T2 phage particles can infect a single bacterium and participate in intracellular growth (Dulbecco, 1949a). The bacterial cell will produce the same number and type of offspring regardless of whether they are derived from one or several parent phage particles. However, the yield of phage may be inhibited if more than 10 infectious units are introduced into a bacterial cell. When two different, but related, phages infect the same cell, offspring of both types and genetic recombinants resembling neither parent may be produced. In mixed infections with completely unrelated pairs, one phage may be dominant and completely inhibit the other (Delbrück and Luria, 1942). The number of phage particles of each type produced in the mixedly infected cell depends on their genetic relationship, which of them is dominant, the multiplicity of each type of infectious particle per bacterial cell, and the times at which infection is initiated with each phage. This phenomenon was originally conceived as inhibition of adsorption and penetration of the superinfecting phage, hence the term mutual exclusion. It is now clear that "exclusion" does not take place at the surface of the host cell but does so intracellularly, *i.e.*, the suppressed phage is "excluded" from multiplying rather than penetrating. The "depressor effect" noted during mutual exclusion simply refers to the fact that the yield of dominant phage is also reduced by the action of the excluded superinfecting phage (Delbrück, 1945). Convincing proof that mutual exclusion and the depressor effect are often, if not always, intracellular phenomena is furnished by studies of induced lysogenic bacteria (Weigle and Delbrück, 1951). If *E. coli* K12, lysogenic for phage *lambda*, is irradiated and then superinfected with T5 phage, the yield of each phage will be reduced appreciably. Obviously, suppression of induced *lambda*, which was inside the cell at the start of the experiment, could not have been caused by its failure to penetrate.

The conditions for demonstrating mutual exclusion (including the depressor effect) of bacterial viruses have been succinctly summarized by Adams (1959): "If a bacterial strain is susceptible to two distinguishable phages, it is possible to study the results of mixed infection of single cells with the pair. If the two infecting phages are not related, the usual result is *mutual exclusion*; one phage or the other multiplies but not both. If one phage is clearly dominant under conditions of simultaneous mixed infection, it is possible to transfer the advantage to the second strain by giving it a few minutes head start. The mechanism of mutual exclusion is not known but it clearly does not involve interference with adsorption, interference with penetration or competition for a unique key enzyme." Except for the time relationships, none of these conditions differentiates mutual exclusion of bacteriophages from interference between animal viruses.

Having cited some of the negative evidence for the mechanism of mutual exclusion, it seems worth while to consider whether the interfering agent can be identified as a constituent of the parental phage particle. It is safe to dismiss the external protein coat of the interfering phage because it does not penetrate the bacterial cell wall (Hershey and Chase, 1952). Ostensibly, we are left with intact phage DNA as the interfering principle, or more accurately, the factor that *initiates* the process of mutual exclusion. This may well be the case, but there are several cogent reasons for examining this hypothesis more closely. The first of these, as previously mentioned, is that prophage DNA, which contains all the genetic information for phage production, may not inhibit multiplication of an unrelated superinfecting phage (Lwoff, 1953; Jacob and Wollman, 1957). This, of course, may be a function of inaccessibility of prophage DNA on the bacterial chromosome. If the lysogenic bacterium is induced, the temperate phage will exclude superinfecting unrelated phage (Weigle and Delbrück, 1951). Of greater significance, perhaps, is the fact that "T2r$^+$ phage heavily irradiated with ultraviolet light is nearly as potent as active phage in stimulating the exclusion of superinfecting phage T2r" (Dulbecco, 1949b). Ultraviolet irradiation damages almost exclusively the genetic material of phage and inhibits its capacity, at least temporarily, to participate in nucleic acid metabolism (Cohen and Arbogast, 1950; Hershey et al., 1954). Therefore, if the DNA molecule of the interfering phage is in fact responsible for inducing mutual exclusion, its inhibitory action cannot be ascribed to production of competing virus DNA by the resistant bacterial cell.

Further presumptive evidence that the infectious component of phage is not identical with its interfering activity is provided by studies with parental phage DNA labeled with P^{32}. Lesley et al. (1951) have demonstrated that DNA of superinfecting phage is rapidly degraded and expelled from the cell. The degradation occurs within a few minutes and is apparently related to enhanced DNase production by the bacterial cell. However, mutual exclusion takes place even if bacterial DNase is inhibited by streptomycin (French et al., 1952) or reduced concentrations of magnesium (Hershey et al., 1954). Under conditions of enzyme inhibition the superinfecting phage DNA is retained within the cell. In addi-

tion, DNA of excluded T2 phage is not incorporated into the progeny of unrelated T1 and T7 phages (French et al., 1952). The studies of Hershey et al. (1954) are of interest in another respect. Their experiments suggest that phage DNA is transferred from parent to offspring in large pieces rather than as constituent nucleotides, whereas the injected DNA of superinfecting phage is degraded in toto.

These studies do not rule out the possibility that phage DNA is the agent responsible for *initiating* the process that leads to mutual exclusion. They merely indicate that the interfering agent within the resistant bacterium cannot be equated with prophage DNA of the bacterial chromosome, degraded DNA, intact DNA components that bear genetic information, or bacterial DNase. Thus, some doubt remains of the validity of the thesis that mutual exclusion depends entirely on genetic or metabolic competition between two incompatible molecules of phage DNA.

Recently published reports suggest a possible alternative approach to a chemical analysis of bacteriophage interference. Hershey (1955) has identified internal protein-like constituents of T2 phage that comprise about 3 per cent of total phage protein. Unlike the external proteins of the head and tail, the internal components penetrate susceptible cells along with phage DNA. Further analysis (Hershey, 1957) reveals at least two internal protein-like substances, one a polypeptide formed by incorporation of host cell lysine and the other, called substance A, which is derived from precursor arginine. Substance A is incorporated into phage progeny, is dialyzable and can enter bacterial cells in the absence of phage DNA. Levine et al. (1958) have shown that an internal protein of disrupted phage is heat stable and immunologically distinct from proteins which comprise the external coat. Of the greatest interest is the finding of Ames et al. (1958) that T4 phage contains substantial quantities of the polyamines putrescine and spermidine derived from host putrescine. It appears that these polyamines may be identical to Hershey's substance A and can combine with phage DNA, presumably by reacting with negatively charged phosphate groups of the DNA molecule. In addition, a different polyamine, spermine, is present in salmonella phages PLT-22 and 98.

With these studies in mind, Levy and Wagner

(1959, *unpublished data*) have attempted to interfere with multiplication of phage T7 by pretreating *E. coli* B with "shockates" of purified T2 phage. Thus far, these efforts have been unsuccessful, possibly because the interfering agent may be the lysine-containing basic polypeptide component of the internal phage protein rather than the polyamines. The fact that the free polypeptide does not adsorb on bacterial cells (Hershey, 1957) poses the difficult problem of designing an experimental model to study its potential interfering activity. Nevertheless, this very tentative postulate, that internal phage proteins may participate in the process of bacteriophage interference, warrants future consideration. Possibly DNA or ultraviolet-irradiated DNA of the interfering phage stimulates the bacterial cell to produce polyamines or basic polypeptides. These compounds within the resistant cell may then combine with and inactivate DNA of the superinfecting phage.

III. INTERFERENCE BETWEEN ANIMAL VIRUSES

It is a rather remarkable fact that so much information about the kinetics of viral interference in animal cells was obtained prior to availability of an adequate experimental model. Until the recent advent of refined cell-culture methods, the only host systems available for semiquantitative studies were the intact chick embryo and Maitland-type cultures of embryonic tissues. The chief drawback of these experimental models is the inability to determine the exact number of cells available for infection and the exact number of virus particles that participate in production of progeny. Nevertheless, several ingenious estimates have been made by direct counting of allantoic cells (Fazekas de St. Groth and Cairns, 1952) and of influenza virus particles (Isaacs, 1957). It is a tribute to the pioneers in this field that much of their data on interference between animals viruses is being substantiated by cell-culture methods.

A. Site of Viral Interference

The first problem confronted by virologists interested in the mechanisms of the interference phenomenon was whether the primary reaction took place at the cell surface or intracellularly. The solution may seem obvious in retrospect but for some time the issue was clouded by the known action of influenza virus on erythrocyte receptors.

It seemed plausible to consider that receptor-destroying enzyme (RDE) might by the responsible factor, despite evidence that only slight and transitory resistance to infection can be produced by excessive quantities of RDE obtained from cholera filtrate (Stone, 1948). Isaacs and Edney (1950) demonstrated that heat-inactivated virus devoid of RDE is an effective interfering agent, whereas formalinized virus with enzymatic activity is not. Similarly, incomplete influenza virus (noninfectious hemagglutinin derived from HeLa cells) may retain receptor-destroying activity despite a markedly reduced capacity to initiate interference (Paucker and Henle, 1958). Schlesinger (1951) has also shown conclusively that destruction of surface receptors by influenza virus does not account for its capacity to interfere with eastern equine encephalomyelitis (EEE) virus. Resistance to the toxic action of influenza viruses induced by cholera filtrate (Wagner, 1952) proved to be completely unrelated to its enzymatic activity (Groupé et al., 1954) and is almost undoubtedly attributable to its content of bacterial endotoxin. It seems clear, parenthetically, that endotoxin-induced resistance to viral infection bears only superficial resemblance to the interference phenomenon (Wagner et al., 1959).

It is, of course, conceivable that interference between myxoviruses may represent alteration of cellular receptor sites by a mechanism other than enzymatic destruction. Baluda (1959) is the chief proponent of the thesis that interfering virus prevents adsorption of superinfecting homologous virus on the surface of host cells. Almost all other investigators have presented evidence to the contrary. To cite but a few examples, Henle et al. (1947) and Isaacs and Edney (1950) have shown that superinfecting virus adsorbs on "interfered" cells and disappears. By implication, it can be assumed that both interfering and superinfecting virus penetrate the resistant cell. The effectiveness of influenza virus as a homologous interfering agent is readily demonstrable even when it is administered *after* infection is established (Henle and Rosenberg, 1949). Moreover, Levine (1958) could detect no difference in the degree to which western equine encephalomyelitis (WEE) virus was adsorbed on susceptible chick embryo cells or on those rendered resistant to superinfection by prior exposure to Newcastle disease virus (NDV). Therefore, unless the interfering agent prevents virus release from infected cells, for

which result there is no evidence, its inhibitory action must be on the intracellular phase of virus multiplication (Henle, 1950).

It is appropriate at this point to examine some of the evidence for interaction between two viruses within a single cell. Baluda (1957), among others, has shown that at least one virus particle per cell is required to induce interference. However, more than one infectious unit can enter a cell. If the infecting dose is excessive, the yield of infectious virus will be diminished. The progeny resulting from large inocula of infectious influenza virus is often composed of a preponderance of noninfectious incomplete virus (von Magnus, 1951). Although there is no assurance that only multiply infected chick allatoic cells can produce incomplete virus (Fazekas de St. Groth and Graham, 1954), it is almost certainly true that the yield of noninfectious hemagglutinin from HeLa cell cultures depends on the number of virus particles that infect each cell (Henle et al., 1955).

Genetic studies furnish more conclusive evidence for interaction of two animal viruses within a single cell. In mixed infections of chick embryo cells with two distinguishable myxoviruses, some of the resultant progeny may be genetic recombinants (Burnet and Lind, 1951) or phenotypically mixed heterozygous variants (Granoff, 1959). Cross reactivation of two viruses rendered noninfectious by ultraviolet irradiation has also been demonstrated (Gotlieb and Hirst, 1956). It seems unlikely that these phenomena can be attributed to anything but double infection of a single cell. Except for difficulty in demonstrating *reciprocal* recombination of influenza viruses (Hirst and Gotlieb, 1955), the analogy to genetic interaction and phenotypic mixing of bacteriophages is clearly apparent.

The often demonstrated fact that a doubly infected cell can produce progeny resembling neither parent has raised the interesting possibility that interference might at times be illusory. Presumably, genetically recombined or phenotypically mixed viruses could be formed which are incapable of infecting test hosts or cell cultures used to detect their presence. However, this seems extremely unlikely as a general occurrence in interference systems. Rates of recombination of related animal viruses are probably very low. Furthermore, the most marked degrees of interference often occur in mixed infections with completely dissimilar viruses which have not been found to undergo genetic interaction.

B. Role of Virus Nucleic Acids in Interference

Let us next consider the possibility that nucleic acids of two interfering viruses might be antagonistic even if they are incapable of genetic interaction. This theory of competitive inhibition of incompatible virus nucleic acids has probably had the greatest vogue. It is potentially supported by the important finding that ribonucleic acids (RNA) of plant and animal viruses are infectious even after their protein coats have been stripped off with phenol (Gierer and Schramm, 1956; Colter, 1958). Not only is the protein coat unessential, but it may actually serve to prevent adsorption of enteroviruses and penetration of their RNA into insusceptible cells (Holland et al., 1959). In addition, Le Clerc (1956) has shown that infected cells treated with ribonuclease have impaired capacities to produce influenza virus, although this effect could conceivably be caused by injury to cellular rather than to viral RNA.

It would be of great interest to learn whether virus RNA can also interfere with virus multiplication. To the writer's knowledge the only evidence that parental virus RNA might be capable of initiating interference is indirect. Notwithstanding, inactivation studies with ionizing and ultraviolet irradiation are not incompatible with the thesis that the interfering property resides in the nucleoprotein fraction of influenza virus (Powell and Pollard, 1956; Powell and Setlow, 1956). Experiments by Tyrrell and Tamm (1955) suggest that 2,5-dimethylbenzimidazole, an antimetabolite of nucleic acid, inhibits the interfering action of heat-inactivated influenza virus. It should be noted, however, that some of their data can be interpreted as showing a direct effect of the benzimidazole compound on the cell as well as on the interfering virus RNA. Of greater significance, perhaps, is the finding that incomplete influenza virus from undiluted egg-passage material had progressively diminished interfering activity and that incomplete virus produced in HeLa cells had no capacity to interfere whatsoever (Paucker and Henle, 1958). Loss of the interfering property is correlated with deficiency of internal S antigen, which, in turn, has been shown to be related to nucleic acid content of influenza virus (Ada and Perry, 1956). Therefore, reasoning by indirection, in order to initiate the

process that leads to interference, an influenza virus particle must contain nucleic acid. Not only is the RNA-deficient incomplete virus an inefficient interfering agent, it is also genetically defective and cannot participate in cross reactivation (Gottlieb and Hirst, 1956).

It is reasonable to assume that competitive inhibition will result if RNA of two viruses should enter the same cell. If, for example, the viruses are genetically related but not identical strains of influenza, the yield of each might be reduced and a small proportion of the progeny could emerge as genetic recombinants or mixed phenotypes. To carry further the analogy to mutual exclusion of bacteriophage, the presence in the same host cell of RNA molecules of two completely unrelated viruses, such as NDV and WEE, should result in a reduced yield of both (Levine, 1958) without genetic crossing. The extent to which the multiplication of each infectious component is suppressed would perforce depend on which virus was dominant in the cell under study, on the ratio of the different types of parental particles in the mixture, and on the interval of time between infection with each virus of the pair. It is only logical to suggest, as does Schlesinger (1959), "that interference in this system may involve direct competition for cellular constituents (or for limited sites?) required for replication of both viruses."

Attractive as it may seem, there is some reason for questioning the validity, or at least the universal applicability, of this thesis. Most of the inconsistencies are also cited by Schlesinger (1959). Foremost, perhaps, is the incontrovertible fact that the infective and interfering properties are differentially susceptible to ultraviolet irradiation (Henle and Henle, 1947). If the inactivated virus particle is unable to impart the genetic information required for production of new progeny, it is difficult to conceive how a sufficient quantity of "interfering" RNA can be formed to compete with the superinfecting virus. It may also be paradoxical that the RNA components of two virus particles can cooperate in the production of recombinant progeny as well as compete with each other. In addition, the capacity of an RNA virus (influenza) to interfere with a DNA virus (vaccinia), cited by Isaacs (1959) as an example of heterologous interference, raises the intriguing question of whether competitive inhibition can occur between nucleic acids with presumed dissimilar metabolic pathways. Also of note is the finding by Schlesinger and Kuske (1959) that the interfering activity of influenza virus is not reversed by treating the "interfered" cells with ribonuclease. The capacity of influenza virus to protect mice against infection with equine encephalomyelitis viruses (Vilches and Hirst, 1947) does not appear to qualify as an example of competitive RNA inhibition as the mechanism of the interference. Influenza virus multiplies in mouse brain largely in an incomplete form (Schlesinger, 1954) and, therefore, the resistant cells are conceivably deficient in "competing" influenza RNA, although these cells appear to produce S antigen not incorporated into incomplete virus. To the reviewer's knowledge no studies have been reported on the capacity of "naked" virus RNA to act directly as an interfering agent. However, Paucker and Henle (1958) made an unsuccessful attempt to render cells resistant to infection by exposing them to internal nucleoprotein S antigen of influenza virus, although, as they point out, there was no assurance that the S antigen even adsorbed on the cells.

The conflicting evidence on the role of RNA in viral interference can, perhaps, best be resolved by postulating that cells form interfering substances other than virus RNA. This hypothesis does not imply that RNA of the interfering virus, either in an intact or noninfectious form, is unessential for *initiating* the processes that lead to cellular resistance. As noted subsequently, soluble interfering substances, distinguishable from nucleic acids, are formed by cells exposed to irradiated and nonirradiated interfering viruses.

C. Interferons

Existing theories of the mechanisms of viral interference must be reevaluated in the light of the discovery of interferon by Isaacs and his associates (Isaacs and Lindenmann, 1957; Lindenmann *et al.*, 1957; Isaacs *et al.*, 1958; Burke and Isaacs, 1958; Isaacs and Burke, 1958; Isaacs, 1959). Interferon, as originally described by these investigators, is a nonsedimentable product formed by interaction of inactivated influenza virus and living cells. When transferred to normal chick embryo cells, it renders them resistant to infection with myxoviruses and vaccinia virus. Reports by other investigators suggest that similar products of infected cells may interfere with the viruses of 17 D yellow fever (Lennette and

Koprowski, 1946), poliomyelitis (Ho and Enders, 1959), vesicular stomatitis (Henle *et al.* 1959), EEE (Wagner, 1959, *unpublished data*), and a variety of other neurotropic viruses (Porterfield, 1959). Rather than attempting to review the published reports, manuscripts in press, and personal communications, the significance of these findings will be evaluated by summarizing some studies on a similar substance currently under investigation in the writer's laboratory. The data are in general accord with the results obtained by others, and represent confirmation and extension of the work of Isaacs and his colleagues.

Our interest in interferon was stimulated by the observation that allantoic fluid infected with the WS strain of influenza A virus had a profound inhibitory effect on multiplication of EEE virus in monolayer cultures of chick embryo fibroblasts. A marked degree of interference between these virus pairs had been noted previously by Schlesinger (1951) and others at a time when quantitative methods for virus and cell assays were not available. We found that infected allantoic fluid containing 10^8 EID_{50} of WS virus completely inhibited the cytopathic effect of 10^9 plaque-forming units (pfu) of EEE virus. The next step was to subject the same infected allantoic fluid to several cycles of high speed centrifugation which reduced its content of influenza virus to 100 EID_{50}. The supernatant of this fluid had exactly the same capacity to interfere with EEE virus as did the original infected fluid. This simple procedure of merely centrifuging infected allantoic fluid to prepare our interferon obviated the necessity for using ultraviolet-irradiated virus and surviving tissue fragments or cell cultures which have been required for preparation of other interferons. Although supernatant fluids from different pools of infected allantoic fluid have varied somewhat in potency, none has been devoid of interferon activity, and the titers as measured by dilution have generally been 10 to 100 times greater than those reported from other laboratories. It should be emphasized that this is probably a reflection of the sensitivity of the assay method in which EEE virus is used rather than other test viruses. Our preparation of interferon does not inhibit plaque formation by NDV in monolayers of the same chick embryo cells, whereas infectious influenza virus does so readily. Isaacs and Westwood (1959) and Porterfield (1959) have recently reported that Arbor viruses

are far more sensitive to the action of interferon than are myxoviruses.

Also of inestimable convenience was the finding that the concentration of interferon is more important than the challenge dose of EEE virus when assayed on chick embryo monolayers by the plaque-inhibition method. No significant difference could be detected in the capacity to inhibit 1 or 10^9 pfu. Thus, we are able to standardize our test system by using a constant input of 40 to 50 pfu, a convenient number for counting. The titer of interferon is read as the 2-fold dilution that reduces the number and size of plaques by approximately half. The error of the assay method on replicate plating is about 50 per cent, which compares favorably with most serologic tests. Of further advantage is the fact that diluted interferon can be added to the test cultures simultaneously with the EEE virus without loss of inhibitory activity, provided that the cell layers are not washed.

It was next incumbent upon us to determine whether interferon is a cell product rather than degraded virus. Four lines of evidence, parallel to those cited by Isaacs, suggest that interferon is not derived from influenza virus particles *per se*: hyperimmune sera with high titers of anti-V (antihemagglutinin prepared in rabbits) or anti-S (soluble CF antibody prepared in guinea pigs) do

TABLE 1

Evidence that interferon is not influenza virus protein

WS Virus Infected Allantoic Fluid	Treatment	Log EID_{50} Titer of WS Virus	Interference Titer*	
			Unheated	65 C
Whole	None	8.0	256	256
Supernatant	Anti-V antibody	<1.0	256	—
Supernatant	Anti-S antibody	2.5	192	—
Supernatant	Red blood cells adsorbed	1.5	192	192
Sediment	Red blood cells eluted	7.5	16	0

* Reciprocal of 2-fold dilution that produces 50 per cent inhibition of 40 to 50 plaque-forming units (pfu) of eastern equine encephalomyelitis (EEE) virus.
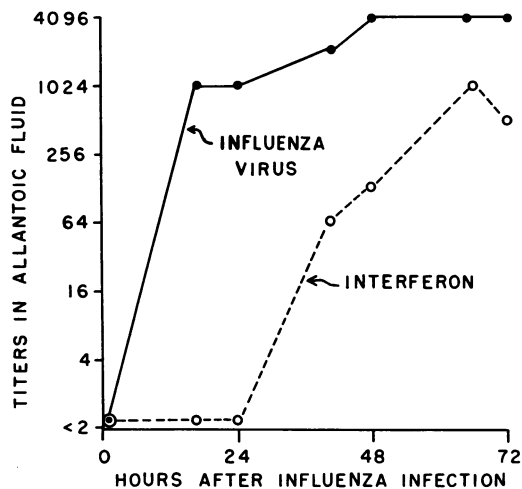
Figure 1. Presence of hemagglutinin and interferon in the allantoic fluid of the same chick embryos at intervals after inoculation with infectious WS influenza virus.

not neutralize interferon activity, interferon does not adsorb on chicken erythrocytes, and the interfering actions of purified influenza virus and semipurified interferon can be differentiated by heat lability. These data are summarized in table 1.

A fourth, and perhaps more convincing, form of evidence was obtained by studying the rate at which infected allantoic cells produce influenza virus and interferon. In this experiment, the hemagglutinin and interferon content of allantoic fluids were measured at intervals after infection with WS influenza virus. Figure 1 demonstrates that large amounts of virus appear in the allantoic fluid before any interferon can be detected. To rule out the possibility that interferon was formed by thermal inactivation of virus *in ovo*, samples of allantoic fluid from the early stages of infection were incubated further at 37 C *in vitro*. This resulted in diminished rather than increased interfering activity of the virus. Thus, it appears from this and the preceding study that allantoic cells infected with influenza virus produce two interfering agents, heat-labile virus and heat-stable, nonsedimentable interferon.

Before interferon could be implicated as an intermediary substance responsible for interference with EEE virus, definite evidence of its intracellular site of action was required. It was readily ascertained that interferon does not in-

activate extracellular EEE virus. This was done simply by demonstrating that infectivity is completely restored following dilution of a noninfectious mixture of EEE virus and interferon. Next, it was found that interferon reacts with host cells. This was accomplished by determining that the rate at which interferon adsorbs on chick embryo fibroblasts coincides, at least approximately, with the rate at which these cells develop resistance to infection with EEE virus. The longer its period of contact with cells, the more interferon is adsorbed and the greater the degree of cellular resistance to virus challenge. After prolonged contact, susceptibility cannot be restored by washing the cells, partial evidence that interferon does not merely adsorb on the surface but penetrates the cell. If the resistant cells are disrupted by alternate freezing and thawing, none of the adsorbed interferon can be recovered. This fact suggests that interferon is rapidly "eclipsed" (metabolized?) and that, unlike virus, it does not stimulate the cell to produce more interferon.

The next question that arose was whether interferon renders cells resistant to infection by altering their capacity to adsorb EEE virus. Although some difficulty was encountered in obtaining reproducible virus adsorption curves, no differences could be detected in the degree to which EEE virus adsorbs on susceptible cells and on cells rendered resistant to infection by treatment with interferon.

Conclusive proof that interferon acts by inhibiting intracellular synthesis of EEE virus was obtained by comparing curves of virus growth in susceptible and resistant cells. In these experiments, cultures of chick embryo fibroblasts were treated with interferon for various periods of

TABLE 2

Effect of interferon on multiplication of eastern equine encephalomyelitis (EEE) virus in chick embryo fibroblasts

Time at Which Cells Were Treated with 256 Units of Interferon	Average Yield of EEE Virus per cell
	*pfu**
Controls (no interferon)........	5000
0 time (simultaneous)..........	25
2 hr before infection...........	2–3
12 hr before infection..........	<1
1 hr *after* infection............	2–10

* Plaque-forming units.

time before or after infection with a multiplicity of approximately 5 EEE virus particles per cell. A summary of the results is shown in table 2. The striking effect of interferon on virus multiplication is clearly evident. These data also support the contention that the inhibitory action is partially dependent on the duration of contact with cells. It also seems safe to assume that exposure to interferon for 12 hr can suppress the virus-producing capacity of almost every cell in the culture and that a significant proportion of the resistant cells are incapable of forming any virus. The most important information derived from these experiments is that interferon affects intracellular virus by inhibiting its multiplication *after* adsorption on cells. Evidence that most of the virus had penetrated the cells 1 hr after infection was obtained by finding that treatment with immune serum did not appreciably reduce the number of infective centers.

Perhaps the most important problem for future study is to determine whether interferon prevents synthesis of EEE virus by direct inactivation of eclipsed parental virus RNA or indirectly alters cellular metabolism of virus nucleic acid and protein. Although we have no satisfactory quantitative data as yet, it does not appear that prolonged exposure to interferon significantly affects the generation time, plating efficiency, or carbohydrate metabolism[2] of resistant cells. It will be of considerable interest to ascertain whether interferon is capable of inactivating infectious RNA of EEE virus separated from its protein coat by phenol extraction (Wecker and Schäfer, 1957).

It is perhaps all too apparent that any further speculation about the mechanisms by which interferon inhibits virus multiplication must be supported by analysis of its chemical nature and reactivity. Unfortunately, only the most preliminary information on the physicochemical properties of interferon is available at present. The results of some of these studies are summarized in table 3. The data indicate that interferon is a protein-like compound, probably of relatively low molecular weight, that is somewhat more stable to heat than the preparation originally described

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[2] The implication of this statement is that interferon does not appear to *decrease* the metabolic activity of the cells. Since this review was prepared, Isaacs (1960 and *personal communication*) has reported *increased* aerobic glycolysis of chick fibroblasts treated with interferon.

TABLE 3

Some physicochemical properties of interferon

Property	Result
Ultracentrifugal sedimentation	Unaffected at 100,000 × G for 4 hr
Dialysis	Not dialyzable
$(NH_4)_2SO_4$ precipitation	90% precipitated at 60% saturation
Enzyme susceptibility	Destroyed by trypsin or chymotrypsin
Enzyme resistance	Not affected by RNase, DNase, papain, plasmin, or RDE
Heat stability	Only 90% destroyed at 85 C for 1 hr; completely stable at 70 C for 1 hr
pH stability	Stable at pH 3–11, > 90% destroyed at pH 12.5, 67% destroyed at pH 1.0
Adsorption on bentonite	Completely adsorbed
Elution from bentonite	Only 25% eluted by pyridine at pH 9 or above
UV absorption spectrum	No UV absorption at any wave length

Abbreviations: RNase, ribonuclease; DNase, deoxyribonuclease; RDE, receptor-destroying enzyme; UV, ultraviolet.

by Isaacs and his colleagues. (A recent personal communication from Isaacs indicates that heat stability is influenced by pH of the suspending medium.) The failure of interferon to absorb ultraviolet light at wave lengths 260 to 290 mμ suggests that it contains no tryptophan, tyrosine, phenylalanine, or nucleic acid constituents. Its marked stability in acid and the fact that it can be eluted from bentonite only at pH 9 or above is consistent with the thesis that interferon is a basic protein or polypeptide. Additional studies indicated that it is not lysozyme and that its biological activity could not be stimulated or blocked by calf thymus histone, RNA, DNA, RNase, or DNase. Therefore, we are left with the *tentative* hypothesis that interferon resembles a basic protein of the histone variety.

It is of interest that basic protein moieties of nucleohistones are biologically much like nucleic acids in their tendency to exhibit species rather

TABLE 4

Species specificity of interferons

Source of Interferons	Interference with Eastern Equine Encephalomyelitis (EEE) Virus		
	Chick cells	Duck cells	Mouse brain
Chick embryo allantois....	+	0	0
Duck embryo allantois....	0	+	NT*
Mouse brain.............	0	NT*	+

* NT = not tested.

than organ specificity (Brachet, 1957). Studies of the specificity of interferon prepared in various species of animals furnish additional *circumstantial* evidence of its biological resemblance to histones. In these experiments chick embryos, duck embryos, and mice infected with WS influenza virus served as three different sources of interferon. Infected allantoic fluids and mouse brain suspensions were centrifuged at 100,000 × G to sediment most of the influenza virus and the supernatant fluids were then tested for their capacity to interfere with EEE virus in chick or duck embryo cell cultures. Comparative studies have not yet been made in mouse embryo cells because they do not support multiplication of the chick-adapted strain of EEE virus used in these experiments. A general pattern of specificity of interferons prepared in different animal species is shown in table 4. These findings are closely analogous to those recently reported by Isaacs and Westwood (1959) who found little, if any, cross resistance to vaccinia infection of rabbit kidney or chick membranes treated with interferons prepared in the heterologous species.

Mention should be made of the difficulties encountered in demonstrating the effect of interferons in intact animals. In the foregoing experiments, definite but only slight resistance to cerebral infection with EEE virus could be elicited by pretreatment with mouse brain interferon. Isaacs *et al.* (1958) noted limited protection against vaccinial infection of chorioallantoic membranes of intact chick embryos previously injected with interferon. Death of chick embryos infected with EEE virus can also be prevented by treatment with interferon, but the most potent preparations afforded only a 30-fold reduction of LD_{50} titer of the challenge virus (Wagner, 1959, *unpublished data*). By comparison, chick embryo cell cultures

could readily be made to resist 10^9 pfu of EEE virus. This discrepancy can undoubtedly be attributed to considerable dilution of interferon in the extraembryonic and extracellular fluids of the intact host, and to the large number of cells potentially susceptible to infection.

Despite the technical difficulties inherent in such an approach, the observation that interference can in fact be "passively transferred" to intact animals may be of considerable theoretical significance. These studies call to mind the question (Schlesinger, 1959) whether viral interference is purely a local cellular phenomenon or can occur at a tissue site distant from a primary infection with interfering virus. Burnet and Fraser (1952) addressed themselves to this question in their studies of resistance to cerebral infection in chick embryos. They found that prior allantoic infection with influenza virus prevented cerebral hemorrhages in embryos challenged intravenously with neurotropic influenza virus. Noting that embryo brain tissue contained an insufficient amount of interfering virus to account for the protective effect, they postulated the existence of a "limiting factor" in the circulation of the resistant embryos. In retrospect, this "limiting factor" could be interferon, which, being a much smaller molecule than the influenza virus, could conceivably pass more readily from the allantoic cavity to the circulating blood and thence to cerebral blood vessels. Confirmation of these hypotheses is being sought in this laboratory (Hook and Wagner, 1958; Grossberg, Hook, and Wagner, 1959, *unpublished data*). We have come to the same conclusion expressed by Burnet and Fraser: that resistance to hemorrhagic encephalopathy in chick embryos cannot be explained solely in terms previously considered as "classical" interference between two viruses infecting the same cells. Although certain technical difficulties must still be surmounted before obtaining conclusive proof, it appears from preliminary studies that allantoic injection of interferon affords chick embryos slight protection against cerebral hemorrhages caused by intravenous injection of neurotropic influenza virus. Gledhill (1959 and *personal communication*) also is seeking to determine by passive transfer studies whether interference with ectromelia by mouse hepatitis virus is caused by the presence of an interferon in the blood of resistant mice.

D. Resistance to Superinfection of Persistently Infected Cell Cultures

Virologists have been intrigued by the knowledge that animal viruses can persist in tissues or cell cultures for long periods of time without producing overt manifestations of infection. The question as to whether some of these persistent infections represent examples of true lysogeny remains unanswered. The conditions for establishment of persistent viral infections vary considerably but often depend on factors such as virulence of the virus (Sabin, 1954), temperature of incubation and growth rate of cells (Bang *et al.*, 1957), and the presence in the supporting culture media of antibody or other antiviral substances (Ginsberg, 1958). More pertinent to the present discussion is the report by Chambers (1957) that persistence of WEE virus in cultures of L cells could not be attributed to alterations of the virus or the cells, or to environmental factors. It is her contention that this chronic infection can best be explained by autointerference.

Persistence of myxoviruses in stable cell lines and resistance of these cultures to superinfection have been the subjects of a comprehensive series of reports published from Henle's laboratory (Henle *et al.*, 1958; Bergs *et al.*, 1958; Deinhardt *et al.*, 1958). Their findings are deemed to be particularly pertinent to this discussion of the interference phenomenon and will be summarized briefly. The chronicity of the infections with myxoviruses is illustrated by the failure to affect a "cure" even after long exposure to immune serum, although specific antibody does suppress the virus temporarily. The cells in these persistently infected cultures grow and divide somewhat more slowly than uninfected cells and exhibit increased aerobic glycolysis and concomitant accumulation of lactic acid (Green *et al.*, 1958). However, these factors do not explain an extraordinary degree of resistance to superinfection with vesicular stomatitis virus (VSV). The latent myxovirus itself does not exhibit any significant change in its biological properties, nor do uninfected clones from these chronically infected cultures. VSV readily adsorbs on and penetrates resistant cells, indicating that their failure to support multiplication of superinfecting virus cannot be attributed to alteration of surface receptor sites. The most interesting fact is that the amount of myxovirus present in the chronically infected cultures is insufficient to account for re-
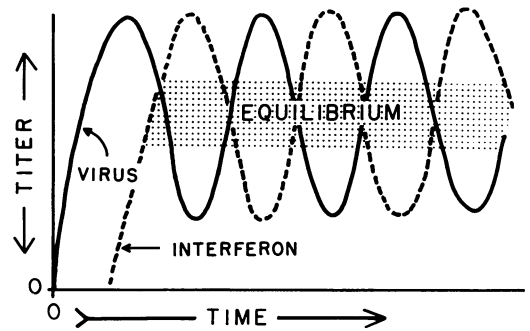

Figure 2. Theoretical schema of the potential equilibrium between virus and interferon produced by cells in a persistently infected culture. Infected cells are constantly being replaced by growth of susceptible uninfected cells at a reduced rate. If the production of virus outstrips that of interferon, all the susceptible cells will die. If interferon production is greater than virus multiplication, the infection will be cured.

sistance to superinfection. Fewer than 10 per cent of the cells contain even a single myxovirus particle at any one time and no incomplete virus can be detected. Therefore, it is necessary to postulate the existence in these cultures of an interfering agent other than the persistent myxovirus itself. A soluble substance, produced in cell cultures after exposure to myxoviruses, that inhibits multiplication of VSV and other viruses has recently been identified in Henle's laboratory as an interferon (Henle *et al.*, 1959; and *personal communication*).

Henle and his colleagues should not be held accountable for the following interpretation of their findings, for which the reviewer takes full responsibility. It seems entirely possible that a cell infected with influenza virus can produce either new virus progeny or interferon, or both. Thus, an equilibrium might be established in the persistently infected culture between these two antagonistic products of cell infection. If the virus is temporarily in the ascendancy, it may stimulate certain cells to produce interferon. If the rate of interferon formation becomes excessive, it might result in a decreased virus titer, thus removing the stimulus for further production of interferon. Consequently, the concentration of interferon, which is not a self-replicating substance, should decline and the virus increase. In this way it is conceivable that a persistent low grade infection can be established in cell cultures

by virtue of cyclic production of both virus and interfering substance in response to viral infection. A diagrammatic representation of this hypothetical situation is shown in figure 2. Obviously, this theory would be subject to wide revision should further studies of persistent infections of cell cultures reveal complete absence of interferons in fluid *and* cellular phases of the cultures. Thus far, however, there seem to be no obvious inconsistencies in studies of persistent infections of cell cultures with other viruses.

IV. SUMMARY AND THEORY

Interference appears to be one mechanism by which the bacterial or animal cell can defend itself against viral infection. It is unlikely that resistance to superinfection takes place at the surface of the cell, but it almost certainly does so intracellularly. There is insufficient evidence to implicate genetic or metabolic factors as explanations for competitive antagonism between nucleic acid moieties of two viruses within the same cell. However, the nucleic acid of the interfering virus may well be essential for initiating the cellular response that leads to interference. If this be the case, the virus contains in its nucleic acid the potential information for its own destruction, mediated by the cellular defenses of the host. Certain interfering viruses stimulate the cell to elaborate protein-like substances of nonviral origin that prevent superinfection with homologous or heterologous viruses. These substances, the interferons, can be secreted by an infected cell and transmitted to other cells, thereby rendering them resistant to infection. Presumably, similar events can transpire in a persistently infected cell culture or an intact animal. It can be predicted with confidence that a considerable amount of future research will be directed toward giving the host an added advantage by passive transfer of the antibiotic-like interfering substances, the interferons.

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DISCUSSION

Referring to figure 2 it was indicated that Tyrrell (Salisbury, England), has demonstrated the phenomenon of repeatedly emerging and disappearing cytopathic virus production in mouse kidney tissue culture infected with WS influenza virus (Westwood, Porton, England).

A factor inhibiting the cytopathic effect of several viral strains has been demonstrated in culture fluids of human kidney cells infected with a chick embryo adapted strain of Type II poliovirus. This inhibitor, or type of interferon, is separable from infective virus and may be a determinant in chronic cell infection *in vitro* and *in vivo*. The development of chronic infection caused by such viruses may be associated with the so-called "zone phenomenon," referring to the ability of certain viruses to proliferate and cause cell destruction when in low concentration but not when in high concentration. For example, the type II poliovirus adapted to chick embryo (RMC virus) causes destruction of human amnion cells in high dilution. Undiluted virus, however, does not destroy the cells but produces chronic cell infection (Ho, Pittsburgh) (Ho, M. and Enders, J. F., An inhibitor of viral activity appearing in infected cell cultures, Proc. Natl. Acad. Sci. (U. S.), **45**, 385–389, 1959).

In considering virus interfering substances there are two factors that are active; one is the virus itself, which is heat labile and can be separated from interferon, which is heat stable (Wagner, Baltimore).