INTERFERONS AND HOST RESISTANCE: WITH PARTICULAR EMPHASIS ON INDUCTION BY COMPLEXED POLYNUCLEOTIDES

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INTRODUCTION

Before 1957 the phenomenon of viral interference, the interference of one virus with the replication of a second, had been observed by several investigators.[1-3] However, it was in that year that perhaps the greatest single step toward understanding this phenomenon was described by Isaacs and Lindenmann.[4] Through an elegantly simple experiment, they discovered that chick chorioallantoic membrane produced a soluble antiviral substance when exposed to heat-inactivated influenza virus. They further demonstrated that pretreatment of fresh chick tissue with this soluble material resulted in development of interference with virus production upon subsequent infection. Isaacs and Lindenmann named this antiviral substance "interferon."

In their early subsequent communications, Dr. Isaacs and his collaborators outlined the general properties which appeared to characterize interferon.[5] These included the fact that interferon was nondialyzable and thus a large molecule, and yet not of large particulate size such as the inducing virions. The interferon-mediated antiviral activity could also be destroyed by exposure to proteolytic enzymes, was stable over a wide pH range, and resisted destruction by exposure up to 60°C. Furthermore, interferon derived from cells of one species was primarily active only in cells of the same species, but inactive in cells derived from tissue of heterologous species. In addition, interferon stimulated protection against infection not by direct inactivation of the invading virus particles, but instead by alteration of the capacity of the cells to support a complete viral replicative cycle.

In the 14 years which have elapsed since Isaacs and Lindenmann's first disclosure, a broad interest in interferon has developed among both investigators concerned with basic mechanisms in biology as well as those more concerned with the search for antiviral materials useful in human and veterinary medicine. The principal areas of investigation have dealt with:

1. More sensitive and meaningful assays of interferon and interferon mediated resistance,
2. The purification and characterization of interferon,
3. A search for interferon inducers and their mechanism of induction,
4. The mechanism of action of interferon in altering cell resistance,
5. The relation of interferon to recovery from virus infections.

It is the intention of the authors to review some of these areas. However, a complete review of the literature related to interferon will not be attempted. Instead, an attempt will be made to highlight the development of existing knowledge with those contributions which have been the guidelines of progress and, further, to detail the most recent advances that have occurred in this rapidly expanding field.

PURIFICATION OF INTERFERON

Since the discovery of interferons by Isaacs and Lindenmann,[4] many investigators have undertaken the task of purification of these substances from various sources. The reasons for attempts at purification may be listed as follows:

1. The remarkable broad antiviral spectrum of the substance made its use as an antiviral agent very attractive.
2. Highly purified interferon would be desirable in a study of its mechanism of action, and also for toxicologic evalution in cell culture and in the whole animal.
3. It would be important to determine the structure and composition and to assess the possibilities of synthesis of a molecule of such remarkable properties.

Chick Interferons

From the original publications of Isaacs and his associates,[5] it was known that the interferon of chick origin contained protein or was at least associated with a protein that was not sedimentable and would not pass through dialysis tubing. The activity could be salted out by ammonium sulfate and was inactivated by treatment with proteolytic enzymes.

Much of the early work was done with chick interferon and in general did not lead to preparations of high purity. It should be stated at the outset that any comparison of specific activities, i.e., the units of antiviral activity per milligram of protein, achieved by various methods of purification, must be interpreted with caution. The biological methods of assay vary so greatly from laboratory to laboratory that any hard comparison might involve only a "numbers game." Fantes,[6] in reporting the results of a method of purification arriving at a specific activity of 2 x 10^6 units/mg protein, stated in addition "by our admittedly rather sensitive assay." Despite the difficulties of variation in biological assay, much progress has been made in the purification of interferons and a better understanding of their properties has been achieved.

Much of the early work on purification of chick interferons did not result in preparations with high specific activity.[7] The first preparation of chick interferon with a high order of purity was achieved by Lampson et al.[8] Influenza virus infected allantoic fluid was the starting material. An overall purification factor of 4500 was obtained with an average specific activity of 235,000 units/mg protein. The material purified by Lampson's method served to answer the following questions:

1. Chick interferon was a highly active protein substance with activity at a level of 0.0042 μg, in protecting chick cells against Eastern equine encephalitis virus.
2. The molecular weight of the major component was found by the biological activity-sedimentation method of Hogeboom and Kuff[9] to be 20,000 to 34,000.
3. The purified material was rapidly destroyed by ultraviolet light contrary to reports by Zemla and Vilcek[10] and Isaacs and Burke[11] reported for crude interferon.
4. The purified chick interferon did not uncouple oxidative phosphorylation and had no detectable effect on the respiration of isolated mitochondria from chicken liver contrary to a report by Isaacs et al.[12]
5. There was no effect on aerobic or anaerobic glycolysis of Ehrlich ascites cells in vitro.
6. The purified interferon did not affect glucose utilization or lactic acid accumulation in chick embryo cells in culture.
7. At this level of purity there was no evidence for the presence of nucleic acid.
8. The overall yield of purified material was extremely low approximating less than 7% of the initial activity present in the allantoic fluid.

Merigan et al.,[13,14] also starting with infected allantoic fluid and using a modified Lampson method, achieved a 6500-fold, and then a 10,000-fold, purification. Bodo[15] started with infected allantoic fluid and, by modifying and using the Lampson and Merigan techniques, arrived at a final 8000-fold purification by

incorporating a final step of filtration through Biogel P-2000.

Perhaps the best purification of chick interferon from allantoic fluid was reported by Fantes.[6] The methods used by this investigator were designed to take advantage of some interesting properties of chick interferon. He found that chick interferon was soluble in some organic solvents such as methanol, ethanol, and acetone, but unlike other proteins with such properties, interferon was also soluble in acid thiocyanate or iodide solutions. Fantes also took advantage of the adsorptive qualities of micronized sodium-aluminum silicates for interferon. By combining these principles with chromatography on DEAE cellulose and CM-sephadex, he achieved a 20,000-fold purification with a specific activity of 1.6 x 10^6 units/mg protein. Fantes[16] was then able to purify chick interferon from cell culture fluid to about the same specific activity. The overall yield was about 7%. He further obtained evidence that even this preparation was largely impure and stated that chick interferon will be, if ultimate purity is achieved, one of the most biologically active substances known.

Multiple Molecular Species of Interferon

Merigan[17,18] reported two molecular species of interferon as present in mouse cell culture. Lampson et al.[19] found two distinct species of interferons in mouse serum differing in both molecular size and in isoelectric point when Newcastle disease virus was used as an inducer. Apparently this occurrence of multiple molecular species is a common phenomenon and is dependent on the animal or cell system, the nature of the inducer, and the time of harvest of the interferons. These interferons also may vary with respect to heat and acid stabilities. In general, it may be said that the mode of action is the same with respect to species specificity (an exception should be noted).[20] It has been shown that all the interferons of a mouse system, prepared in vitro or in vivo of whatever molecular size, and induced by viral or non-viral substances, could be neutralized by an antiserum prepared to mouse cell culture interferons induced by a virus.[21] It is not now known whether these interferons are complexes of monomers, represent aggregates, are bound to nonspecific carriers, or are completely separate entities. These questions remain to be answered by further work.

New Knowledge Concerning Purification and Properties of Mouse and Human Interferons

Much of the latest knowledge concerning purification of interferons is very well covered in reviews by Fantes.[6,22] However, a most noteworthy paper by Carter[23] has appeared with respect to purification of both mouse and human interferons. Carter employed a series of steps involving differential precipitation, centrifugation, gel chromatography, and finally isoelectric focusing in thin layers of acrylamide gel containing carrier ampholytes. The sources of interferons in both cases were cell culture. Mouse interferon was recovered in yields of 60 to 100% while human interferon recoveries ranged from 12 to 80%. The major purification was achieved by the isoelectric focusing technique. The mouse interferon was purified 593-fold with a specific activity of 4.5 x 10^6 units/mg protein. The mouse interferon appeared in two distinct molecular species with isoelectric points of 7.15 and 7.35 and with unequal specific activities. Human interferons were prepared with three isoelectric species at 5.35, 5.60, and 5.70. Carter further demonstrated that both mouse and human interferons were separable into two distinct forms with respect to molecular weight, one of which was twice the molecular size of the other. He suggested that the larger molecule is a dimer of the smaller molecule. In addition, he suggested that the condition of low ionic strength under which the electrofocusing procedure operates, causes a dissociation of the interferons. This latter point was shown to be true by simple dialysis versus low ionic strength salt solution followed by gel chromatography, where the smaller molecular weight molecule was recovered from the column. The monomeric unit of mouse interferon was 19,000 daltons while the monomeric unit of human interferon was 12,000 daltons. Despite the high specific activity reported by Carter, he does note that the purification of interferons through the steps he outlined led to problems of instability. This work by Carter will give much impetus to the continuing effort to achieve ultimately pure human interferon for studies of structure and composition with a view toward possible synthesis of the molecule.

THE ACTION OF INTERFERONS

Perhaps the two most outstanding biological characteristics of interferons are 1) the inhibitory

effect against a broad range of both DNA and RNA viruses and several other intracellular parasites without corresponding detectable inhibitory effect upon the host cells and 2) the narrow range of protective activity affecting cells from the same or closely related animal species. In studying the effect of interferon on cells and virus replication in order to arrive at detailed mechanisms by which interferon might stimulate cellular resistance, these two characteristics must be thoroughly explained. To date, a great deal of data has been accumulated to more accurately define these characteristics, and these data are summarized in the following paragraphs. The central explanations have, as yet, remained elusive. This leaves only a few proposed and highly controversial potential mechanisms.

Organisms Affected by Interferons

Some members of virtually all groups of viruses have been shown to be susceptible to the action of interferons.[24,25] However, the degree of susceptibility has varied greatly. Arboviruses have appeared to be the most susceptible group,[26] while adenoviruses have been only poorly sensitive.[27] Viruses having intermediate sensitivity have included vesicular stomatitis and vaccinia.[28] As might be expected, the development of any graded list such as this suffers from the lack of consistency of data from laboratory to laboratory. Generally, the interferons tested have been crude or only partially purified preparations, and the studies have been carried out with interferons from a variety of sources, tested in different cell systems, and by varying assay techniques.[28] Since a variety of separable interferons have been described,[16] it seems reasonable to question any comparisons of virus susceptibility to interferons unless those comparisons have been made using purified interferon preparations under standardized assay procedures. The general assumption that all interferons have a similar range of antiviral activity has yet to be fully substantiated.

Some well-documented differences in virus susceptibility have been noted. Wagner[24] reported that at least 1000 times more interferon was required to inhibit Newcastle disease virus (NDV) than to inhibit Eastern equine encephalitis virus. A similar finding was also reported by Lockart[26] with NDV and Western equine encephalitis viruses.

Several other groups of nonviral intracellular parasites have been demonstrated to have sensitivity to interferons. Hanna and associates demonstrated interferon activity against TRIC agents, members of the psittacosis-lymphogranuloma venereum group of organisms. Remington and Merigan[30] have demonstrated protection of cells against infection with *Toxoplasma gondi*, and Jahiel[31] has reported similar protection against *Plasmodium berghei* malaria in mice.

Host Specificity of Interferons

One of the most frequent observations made concerning interferons has been the specific activity in cells obtained from animals of the same species from which the interferons were produced.[32-34] This observation has usually been used as one of the criteria for the definition of an interferon and has been made with partially purified as well as crude interferon preparations. Partially purified chick interferon was active in protecting only chick cells.[8,13,35] Partially purified mouse interferon was active in protecting only mouse cells.[17,18] There have also been occasional reports of protective activity by interferons on cells from a heterologous species. Without using highly purified interferon, the presence of other non-interferon contaminant virus inhibitors must be considered. The presence of such inhibitors has been described by Buckler and Baron[36] and Paucker.[37] Some notable exceptions to strict species specificity have been observed and should be discussed more fully.

Desmyter et al.[20] described interferon prepared in human foreskin cells which was 20-fold more active in protecting rabbit cells than human cells against vesicular stomatitis virus (VSV) infection. The properties of the activity in heterologous and homologous cells were those of interferons. Levy-Koenig et al.[38] demonstrated that interferons induced by ultraviolet irradiated NDV in human foreskin, amnion, embryonic spleen, and leukocytes all protected rabbit kidney cells at least as well as human foreskin cells against VSV challenge. The heterospecific protective activity of human interferons was neutralized by antisera prepared against rabbit and mouse cell interferon, indicating that perhaps common antigens are shared by these interferons.

Bucknall[39] used interferons prepared by infecting either human leukocytes or monkey *Macaca nemestrina* leukocytes with Sendai virus.

The crude interferon preparations (in which infectious virus was destroyed) were tested for capacity to protect homologous and heterologous cells against virus infection. Interferon prepared in Macaca leukocytes was protective in primary kidney cells derived from either genus of monkeys (*Cercopithecus aethiops* and *Erythrocebus patas*) as well as in human embryonic lung cells. Similarly, the human interferon preparation was active in cells derived from either monkey genus. Neither monkey nor human interferon conferred protection on mouse L cells. All protective activities were destroyed by trypsin treatment, but not by incubation at pH 2 or by treatment with ribonuclease. Although these interferon preparations were crude, the suggestion of not only species crossover of activity but also genus crossover appeared to be undeniable. A more detailed analysis of range of protective activity of highly purified primate interferons to determine existence of "species," "genus," and "family" crossover could possibly have practical significance for potential interferon therapy in man.

Moehring and Stinebring[40] examined the range of protective activities of crude avian interferons produced in ovo or cell cultures using influenza A-1 virus and ultraviolet-treated Newcastle disease virus, respectively. A great deal of cross-protective activity was found for interferons produced by members of the order *Galliformes* (chicken, pheasant, chukar partridge, bob-white quail, guinea foul, turkey). None of the *Galliformes* interferons were active in members of order *Anseriformes* (Pekin duck, goose) or *Columbiformes* (common pigeon). Similarly, interferons from *Anseriformes* could protect members of this same order, but not members of the orders *Galliformes* or *Columbiformes*. Thus, in agreement with Bucknall's data for primate interferon, "order specificity" but not "species specificity" seemed to prevail. In addition, one-way cross-sensitivity similar to that reported for human interferon[20] in rabbit cells was observed for interferons from members of the order *Galliformes*. Interferon from all members of *Galliformes* could protect quail cell cultures as well as the homologous cell cultures, but quail interferon was only active in the homologous quail cells.

From these reports, it would appear that the "species specificity" originally described early in interferon research may have to be redefined. At present, we can conclude that a narrow range of protective activity for homologous and closely related cells exists. Rather than "species specificity," "order specificity" may more closely define the selective sensitivity of cells. The "one-way" crossing of activity beyond the boundaries defined by species or order, as demonstrated for some human interferons, must be taken into account. The ultimate explanation of the limited range of protection of cells must await use of highly purified interferons in well-controlled cell culture system.

Cell-Interferon Interaction

Early interferon research, in which crude interferon preparations were used, indicated that treatment of cells may alter cell morphology, cell growth rate, cellular RNA metabolism, and aerobic glycolysis.[12,41-43] Data obtained using more highly purified interferons failed to substantiate these observations. Purified chick interferon failed to alter the rate of aerobic glycolysis[8] and did not alter protein or RNA metabolism of treated chick cells.[44] Cocito et al.[45] further demonstrated that the toxic effects of crude interferons on cells could be accounted for by contaminants which could also be extracted from normal cells. The only consistently observed alteration of cells exposed to purified interferons has been the development of resistance to virus infection.

It is, as yet, quite unclear what occurs initially upon exposure of cells to interferons. Numerous studies designed to determine the uptake of interferon by cells have been carried out using both crude as well as partially purified interferon preparations.[13,46,47] Since these studies utilized changes in the amount of interferon activity that remained in preparations used to treat cells, any conclusions must be carefully qualified. The apparent uptake[47] could be due to extracellular destruction of interferon without adsorption to cells, or nonspecific adsorption to cell debris. Similarly, the apparent lack of uptake[46] could be due to continuous production of new interferons in treated cell cultures from contamination of interferon preparations with potent interferon inducers such as double-stranded RNA.[48] Consequently, as was the case in studies of species specificity, definitive conclusions about interaction of cells with interferon must await specific physical, chemical, or serological techniques to identify the interferon molecules. The development of purified radioisotope labeled

interferon or antibodies specific for the interferon molecule could lead to direct evidence of uptake rather than the indirect evidence so far accumulated.

Development of Resistance in the Interferon-Treated Cell

Friedman[49] has demonstrated that specific binding of interferon to the cell surface must occur in order for subsequent development of resistance. Chick cells exposed to interferon for several hours at 1°C developed resistance to virus challenge following further incubation at 37°C, after removal of excess, unadsorbed interferon by washing. The degree of resistance was proportional to the concentration of interferon used and to the incubation time at 1°C. Exposure of cells to trypsin following incubation with interferon at 1°C blocked subsequent development of resistance upon temperature elevation to 37°C. It appeared that interferon did bind to cell surface sites at 1°C, but further development of resistance required temperature elevation. In addition, it appeared that interferon must be present at the time of temperature elevation since trypsin blocked development of resistance but not the binding sites on cells.

Much of the early research concerned with the action of interferon focused on the effect of alterations in environmental conditions. Isaacs and associates[50] observed that increased oxygen tension diminished the effectiveness of interferon, but later experiments done with greater care showed that oxygen tension had no effect on the action of interferon.[51] Zemla and Schramek[52] demonstrated also that interferon induced resistance to Western equine encephalitis in chick cells incubated under anaerobic conditions. Similarly, there was no clear effect over a wide range of pH on the action of interferon in cell systems.[51,53]

A much more widely used approach to investigation of interferon action has been to employ inhibitors of specific cellular metabolic functions. This indirect approach to investigation of the requirement for certain functions is appealing in its simplicity, but conclusions often have been based on assumed selective activity of the given inhibitor. Although an inhibitor may have but one known inhibitory function, the lack of secondary effects cannot be ruled out. Thus, the data obtained using metabolic inhibitors may indicate, but not prove, the requirement of a specific cellular function for interferon action. The final proof must emerge only from direct evidence wherever possible.

With these qualifying remarks in mind, the accumulated data have indicated that the following metabolic functions are apparently involved in development of antiviral activity in interferon-treated cells.

1. **Nucleic Acid Synthesis** - Taylor[54] demonstrated the dramatic inhibitory effect of Dactinomycin on interferon action, suggesting that DNA-dependent RNA synthesis was required. This demonstration further suggested that other chemical agents which alter the template function of DNA might block the development of interferon mediated antiviral activity. Indeed, mitomycin C, which causes interstrand cross-linking and degradation of DNA,[55] iododeoxyuridine,[56] and 5-mercaptomethyl uracil, both thymidine analogs, inhibited interferon action.[57] Inhibition of DNA synthesis by 5-fluoro-2′-deoxyuridine had no effect on interferon action.[57] Other inhibitors of interferon activity which are mainly inhibitors of RNA synthesis include 6-mercaptopurine,[57] 6-azauridine, 5-fluorouracil,[58] and azacytidine.[59] Aminonucleoside puromycin, primarily an inhibitor of ribosomal RNA synthesis, but not of messenger and transfer RNA synthesis, had no effect on interferon action.[57]

2. **Protein Synthesis** - Both puromycin[60,61] and cycloheximide [57] blocked the development of antiviral activity in interferon-treated cells. Both agents block protein synthesis, but at different sites. Furthermore, the amino acid analog p-fluorophenylalanine inhibited interferon action.[62] More recently, Friedman and Pastan[63] demonstrated that cyclic 3′5′-adenosine monophosphate (CAMP) enhanced antiviral activity of interferon in cell culture. They pointed out that CAMP increases the rate of synthesis of some inducible *E. coli* enzymes and may enhance the antiviral activity of interferon by this same stimulation of synthesis of specific proteins necessary for the expression of antiviral activity.

3. **Phospholipid Synthesis** - Friedman and Pastan[59] demonstrated that under conditions in which neither cellular RNA nor protein synthesis was inhibited, 2-mercapto-(β-4-pyridethyl) benzimidazole (MPB) could reversibly inhibit interferon action. They suggested that the

inhibitory step came after binding of interferon to the cells, but before the required RNA and protein synthesis occurred. Since MPB has been shown to inhibit phospholipid synthesis and other membrane associated phenomena, it was suggested that MPB may block entry of interferon into the cytoplasm by transport from the surface site of attachment.

Inhibition of Virus Replication

Very early in the history of interferon research, it was realized that interferon treatment of cells did not interfere with the adsorption or release of virus particles.[64,65] Furthermore, since interferon could inhibit the infectivity of poliovirus RNA[66] the site of action must occur after penetration and uncoating of virus particles, at some stages of the intracellular replication and maturation of particles. Levy[67] demonstrated that RNA was not synthesized in Mengo virus infected cells previously treated with interferon and thus some early event in viral replication must have been inhibited. This same obervation has been made for Semliki Forest virus.[68] Interferon treatment also inhibited the synthesis of viral-coded RNA polymerases in Semliki Forest virus and Mengo virus infected cells.[69,70] Recent research reported by Friedman[59] has concentrated on the earliest observed inhibited event, the replication of the parental viral RNA genome in Semliki Forest virus infected chick cells. He demonstrated that the normal conversion of input viral RNA into double-stranded replicative form was blocked. This conversion should occur as a result of de novo synthesis of RNA ploymerase and the subsequent synthesis of a minus RNA strand complementary to the input viral RNA genome. The formation of replicative form involves a membrane-bound complex of viral plus strands, RNA polymerase, and newly synthesized complementary minus strands. In the interferon-treated cells, only input single-stranded viral RNA was membrane-associated. Furthermore, Friedman demonstrated that in interferon-treated cells, no new detectable proteins were synthesized from input viral RNA. Thus, the inhibition of both viral RNA synthesis and viral protein synthesis in RNA virus infections could be traced back to the very early inhibition of translation of the viral input RNA.

Could the same explanation account for the inhibitory action of interferon on DNA virus replication? Joklik and Merigan[71] thoroughly investigated the effect of interferon treatment on replication of vaccinia virus in L cells. They observed that in interferon-treated cells, the host cell polyribosomes rapidly disaggregated (as they probably did during normal vaccinia replication). Although synthesis of viral messenger RNA was actually enhanced in interferon-treated cells, no formation of new polyribosomes occurred. As a result, no new viral coded proteins, including DNA polymerase, were synthesized, resulting in no new synthesis of progeny DNA or structural proteins. In the course of this abortive infection, complete cell destruction occurred in five to six hours. These data have subsequently been further explained by the demonstrations that unlike the case described above for RNA viruses, vaccinia virions contain the RNA polymerase.[72,73] In the absence of viral protein synthesis, this polymerase has enhanced capacity to synthesize viral messenger RNA. The destruction of host polyribosomes and inhibition of host protein synthesis were probably due to inhibitory activity associated with the virions.[74] In interferon-treated cells, viral uncoating is also inhibited.[75] This may be a reflection of the requirement for synthesis of a new viral messenger RNA which codes for uncoating protein.[76] Thus, as was the case with RNA viruses, the inhibition of this DNA virus could also be explained on the basis of inhibition of translation of viral messenger RNA molecules.

A somewhat different situation has emerged from studies involving SV40, a small DNA virus. Pretreatment with interferon inhibited the subsequent transformation of cells and production of T antigen, an early viral protein, on infection with SV40.[77] However, for cells already transformed by SV40, interferon had no effect on T antigen formation.[78] Similarly, infection of interferon-treated cells with adenovirus-SV40 hybrid virus also resulted in T antigen formation. However, when interferon-treated cells were simultaneously infected with the separate adeno - and SV40 viruses, T antigen production was inhibited.[79] Thus, the sensitivity of production of this viral protein to inhibition by interferon depended upon the nature of the viral genome. Unintegrated SV40 was interferon sensitive, whereas either cell-integrated or adenovirus hybridized SV40 was interferon-resistant.

Recently, Oxman and Levin[80] measured the effect of human, monkey, and mouse interferon on production of early viral protein (T antigen)

and viral-specific RNA. Both primate interferons, but not mouse interferon, were active in monkey cells, inhibiting both T antigen and virus-specific RNA synthesis. The authors suggested that the interferons may have inhibited transcription of SV40 DNA to form the virus specific RNA molecules. Alternatively, they suggested that some very early gene (proto-early gene) whose transcription is catalyzed by cellular DNA-dependent RNA polymerase may produce a protein which regulates transcription of the remaining early SV40 genes. In this case, interferon could inhibit the translational step from proto-early gene messenger RNA to final regulator protein. This latter interpretation would be consistent with the hypothesis that interferon blocks translation of viral messenger, as suggested from the work with the RNA viruses and vaccinia.

Proposed Mechanisms of Interferon Action

The suggestions, as they have been developed above, are that for interferon-treated cells to develop resistance they must be capable of both RNA and protein synthesis and must develop the capacity to inhibit translation or perhaps transcription of viral nucleic acids. This would suggest that interferon was itself the inducer of some newly synthesized RNA and protein and that the key which could unlock the answer to the mechanism of action of interferon should be found associated with the metabolic machinery involved in protein synthesis. In 1966, Marcus and Salb[81] presented evidence, obtained through in vitro studies, which indicated that compared to ribosomes from untreated cells, ribosomes from interferon-treated chick cells were only poorly capable of binding viral RNA and unable to translate that RNA which was bound. However, the ribosomes from treated cells were quite capable of binding and translating non-viral messenger RNA. Since newly synthesized protein was believed to be required for interferon action, they suggested that this newly synthesized protein was the active antiviral agent and acted by binding and selectively altering the capacity of ribosomes to translate RNA. Thus, they named this hypothetical protein "Translational Inhibitory Protein" or TIP. In 1967, Carter and Levy[82] reached similar conclusions that ribosomes from interferon-treated cells could selectively discriminate between viral and cellular RNA templates by binding with only the latter. This "altered ribosome" hypothesis was extremely appealing to interferon researchers and was generally accepted at the time. The inhibition of viral replication at the translational level appeared to be explained. The need for new macromolecular synthesis could be accounted for by the synthesis of TIP, which also helped to explain the high specific activity of interferon. Furthermore, the "altered ribosome" hypothesis explained the selectivity of inhibition of viral but not cellular functions at the subcellular level. As appealing as the hypothesis has been, it must be accepted only as hypotheis. The selective binding of messenger RNAs by interferon-treated ribosomes has not been generally confirmed and, in fact, extensive studies reported by Sonnabend et al.[83] have failed to confirm the suggestions of Marcus and Salb, and Carter and Levy. No unique binding or translational properties were observed by Sonnabend's group.

Sonnabend et al. [83] have briefly mentioned other possible appealing hypotheses for interferon action. Rather than introducing the concept of TIP, perhaps interferon itself is the antiviral substance. The need for new macromolecular synthesis could involve synthesis of new proteins to facilitate uptake or transport of interferon to the active site within the cell. Alternatively, perhaps, interferon normally exists as an inactive molecule which must be activated to develop antiviral activity. Such an activation process might require synthesis of a new specific cellular enzyme, the synthesis of which would be activated by the interferon. The observations of narrow protective ranges of interferons for cells from related animals could be explained by either the selective uptake, transport, or activation within the cell. The activation of larger precursor polypeptides by specific cleavage is well documented for enzymes and virus capsid proteins. The high potency or specific activity of interferon could be explained if the activation process involved disaggregation of interferon molecules into their active subunits. The suggestion that extracellular interferons exist as molecular aggregates was previously mentioned and has gained support with observations of Carter.[23]

As stated at the beginning of this section, elucidation of the mechanism of action of interferon must involve understanding 1) the selective inhibitory effect against a broad range of intracellular parasites and 2) the narrow range of

protective capacity affecting cells from closely related animal species. At this stage in interferon research, we are still gathering an abundance of facts and have sketched the general patterns of interferon action, but remain far from a detailed comprehensive understanding at the molecular level. Workers in this field must be open-minded concerning potential explanations of the action of interferon and accept an appealing hypothesis, only after a steady accumulation of supportive evidence which fully explains all characteristics of the action of the interferons. Similar openmindedness to the possibility of more than a single mechanism of action of interferons seems equally warranted.

INTERFERONS INDUCED BY VIRUSES

In considering the induction of interferons by viruses, we are confronted with two basic questions. What is the nature of the inducer(s) associated with virus infection which triggers the production of interferons; and what is the mechanism by which cells respond to this inducer to produce interferon? Both questions have been the subject of considerable research leading to a wealth of facts, several hypotheses, and an abundance of controversy.

What is a Viral Interferon Inducer?

Ho[84] has stated, "Practically every major group of virus has been found to induce the production of interferon in a suitable host or cell system." Thus, cells have been confronted with a large variety of chemically and physically different particle types and, yet, under appropriate combinations of cells and particles, interferon production has been triggered. Since a given cell type, such as chick embryo, has been shown to be inducible by either DNA or RNA containing viruses,[8,35] it appears that the genetic message for production of the interferon molecules resides within the cell genome. What is recognized by the individual cell in order to trigger interferon production? Does this diverse group of viral particles possess any common characteristic recongnizable by the cell as an interferon inducer? The candidates for specific interferon inducer are whole virions, virion proteins, virion nucleic acid, or some product of intracellular viral replication.

It is unlikely that whole particles act as interferon inducers. Although numerous animal virus particles do induce interferon production, bacteriophage viral particles[85] do not. Similarly, members of the group A arboviruses, usually excellent interferon inducers, lose their inducing capacity upon sufficient exposure to ultraviolet irradiation to destroy their infectivity.[86] Burke et al.[87] also showed that treatment of Semliki Forest virus with hydroxylamine, under conditions which should have only altered the virion RNA, destroyed the capacity of the particles to induce interferon.

The question of interferon induction by inactivated virus particles has been and still remains controversial. Isaacs[5] suggested that a virus must be inactivated in order to induce interferon. Ho[88] demonstrated that while infectious Newcastle disease virus was incapable of interferon induction, UV-irradiated virus was a competent inducer. However, this inducing capacity was again destroyed by excessive irradiation. Ho[84] has further pointed out that examples of interferon induction without complete viral replication have been observed for Sendai virus, poliovirus, and vaccinia virus. For all these examples, some clarification is necessary.

The lack of interferon induction by infectious viruses has been explored by Gandhi and Burke[89] using influenza and parainfluenza viruses. Unirradiated virus depressed rates of host cell RNA and protein synthesis in the course of infection, thus suppressing production of a new protein-interferon. However, irradiation destroyed this inhibitory effect and interferon was produced.

Generally, "inactivated" virus has referred to the incapacity of virus to carry out a complete replicative cycle resulting in production of infectious viral progeny. Rarely have researchers using "inactivated" virus to induce interferon investigated what viral-directed activities might still occur in the induced cell. To assume that "inactivated" virions are inert virions is incorrect. In 1961 Gresser[90] showed that although Sendai virus did not form complete viral progeny in human amnion cells, interferon, hemadsorption antigen, and cytopathology were developed. Similarly, Huppert, Hillova, and Gresland[91] described the synthesis of virus-specific RNA in chick embryo cells infected with ultraviolet-irradiated Newcastle disease virus, which was incapable of producing infectious virus. Furthermore, as shall be developed more fully below, even virion preparations incapable of partial replication

may be carriers of competent interferon inducers such as double-stranded RNA.

No clear evidence is presently available to indicate that viral proteins induce interferon. Several reports[92,93] that treatment of adenovirus types 5 and 12 and Newcastle disease virus with trypsin destroyed their interferon inducing capacity while not affecting infectivities, while intriguing, are not definitive.

Unlike the situation for viral proteins, a very clear case has been developed implicating virion RNAs or viral-coded RNAs as inducers of interferons. Skehel and Burke[94,95] showed that production of infectious virus, virus hemagglutinin, virus-induced RNA synthesis, virus-induced RNA polymerase, and interferon were all inactivated with first order kinetics upon treatment of Semliki Forest virus (SFV) with hydroxylamine. Since hydroxylamine reacts with viral nucleic acid, they concluded that the nucleic acid was essential for interferon induction. Furthermore, by using temperature shift experiments, they concluded that a small amount of viral RNA synthesis was required in chick cells infected with SFV in order to stimulate interferon production.

Definitive evidence that the nucleic acid contained within the viral capsid is an interferon inducer has been complicated by reports of studies using concentrated but crude or only partially purified virus preparations. Recently, Dianzani[96] demonstrated that high multiplicities of NDV could induce interferon production in L cells under conditions where no evidence of viral replication was detectable. Goorha and Gifford[97] used high multiplicities of heat-treated Semliki Forest virus to produce interferon in chick embryo cells, again under conditions where no viral replication was detectable. They concluded that viral RNA synthesis was unnecessary for interferon induction. Lomniczi and Burke[98] detected interferon production in chick embryo cells subjected to high multiplicity of infection with Semliki Forest virus incapable of synthesizing detectable RNA. These studies indicate that either: a) input viral RNA or other viral component was capable of interferon induction; b) some synthesis of double-stranded replicative-form RNA did occur and was the active inducer; or c) the input virus preparation was contaminated with small amounts of double-stranded RNA. As will be discussed more fully below, such molecules are highly potent interferon inducers and are normally produced during replication of RNA viruses. Single-stranded RNAs have proven very inadequate as interferon inducers and it would be doubtful that input viral RNA would act as an inducer unless it carried sufficient secondary structure to mimic a double-stranded molecule. That some undetected synthesis of replicative-form could have occurred was already discussed and can never be fully dismissed. However, a more likely possibility is that these crude viral concentrates carry double-stranded RNA, perhaps originating as replicative-form RNA produced during the virus replicative cycle. Field et al.[48] recently examined such crude preparations of Newcastle disease virus, Semliki Forest virus, and Sindbis virus and found that nucleic acid extracts contained double-stranded RNA detectable by reaction with antibody specific for double-stranded RNA. Although double-stranded RNA represented a small fraction of the total RNA present, it was capable of induction of viral interference in cell cultures. Thus, investigators of interferon induction under conditions of no dectectable viral synthesis must account for possible contaminating double-stranded RNA in the virus preparation before drawing any conclusion concerning potential induction by other viral components.

Several examples of RNA extracted from purified virions have been documented as interferon inducers. In each case, the virus in question contained double-stranded RNA. These have included Reovirus type 3,[99] rice dwarf virus and cytoplasmic polyhedrosis virus,[100] and mycophages associated with *Penicillia*.[101]

The fourth possibility – that viruses induce interferon indirectly through inducer synthesized during the replicative cycle – has been the subject of some thorough studies. Falcoff and Falcoff[102] carefully examined the products of Mengo virus infection for potential interferon inducers. They observed that a single-stranded RNA extracted from Mengo virus and uninfected L-cells failed to induce interferon. The fractionation of RNA molecules from infected cells revealed that only double-stranded RNA produced during replication was capable of stimulating interferon induction. Furthermore, they demonstrated that of the two forms of viral-coded double-stranded RNA, replicative-form and replicative-intermediate, only the former and the ribonuclease resistant portion

of the latter were capable of interferon induction.[167] Replicative-intermediate RNA is thought to differ from replicative-form RNA in containing uncomplexed strands of single-stranded RNA at intervals along the double-stranded helix. These studies clearly indicated that of all the molecules produced during viral replication, only double-stranded RNA could be indentified as having the capacity to induce interferon. Similar studies of inducing capacities of viral RNA, associated with influenza virus replication[103] and Sindbis virus replication,[104] have indicated that only the double-stranded replicative-form RNAs and not the single-stranded viral RNA can induce interferon.

Colby and Duesberg[103] demonstrated that even for vaccinia virus infected cells, double-stranded RNA with capacity to induce interferon was produced during viral replication. The production of double-stranded RNA during relication of a DNA-containing virus has also been observed for fowlpox and myxoma viruses, and for T_4 and λ bacteriophages.[170] This may represent a general phenomenon for DNA virus replication, and if this were true, it could explain the capacity of DNA viruses to induce interferon.

Thus, these studies, designed to fractionate the virus or virus-infected cell to purify and characterize potential interferon inducers, have strongly suggested that double-stranded RNAs either contained within the virion, as seen for reovirus, or produced during viral replication, do act as the viral-coded inducers of interferon. Skeptics may still suggest that such extracted molecules do not really represent the inducer molecules as they exist under the intracellular condition, and consequently may represent extraction artifacts. However, a recent paper by Stollar and Stollar[105] has provided direct evidence of the existence of double-stranded RNA within virus-infected cells. Such replicative-form RNAs were identified by immunofluorescence using antibody specific for double-stranded RNA.

Another approach which has been utilized to study interferon induction by viruses employed temperature sensitive mutants which prevent either viral RNA or viral protein synthesis under restrictive conditions. Lockart et al.,[106] using mutants of Sindbis virus, and Lomniczi and Burke,[98] using Semliki Forest virus, have each shown that RNA-mutants (incapable of RNA synthesis at nonpermissive temperature) failed to induce interferon. However, Sindbis RNA (+) mutants (capable of RNA synthesis but lacking some vital protein synthesis under restrictive conditions) produced only low interferon yields. Lockart et al. have concluded that some viral processes, perhaps besides RNA synthesis, were necessary for interferon induction. For Semliki Forest virus, Lomniczi and Burke found also that RNA (+) mutants had poorer capacity than wild-type to induce interferon, even though the same amounts of RNA synthesis appeared to occur as in wild-type infected cells. These observations remain unexplained, but do suggest that in virus infected cells, viral RNA synthesis alone is not sufficient to stimulate interferon production. Some other viral-coded process may also be required. This, perhaps, could involve alteration of replicative-intermediate RNA to produce replicative-form RNA or some similar process to allow accumulation of a sufficient amount of double-stranded RNA capable of stimulating the cell to produce interferon.

How do Cells Produce Interferon?

A detailed explanation of the response of the cell to a viral interferon inducer is complicated by the very nature of the inducer. It becomes necessary to study one induction process in a system in which the genetic information for induction of many new processes has been introduced. The lack of purified inducer, unless induction by double-stranded polynucleotides mimics induction by viruses, may never permit the detailed analysis of the isolated interferon induction event. Yet primarily through the use of metabolic inhibitors some understanding of the host cell's response to viral stimulated interferon has emerged. A more detailed survey of the literature has been presented by Burke[107] and by Vilcek[108] and the following analysis is meant only to highlight those findings which, in our opinion, are most important. Virus-induced production of interferon appears to require functional DNA within the host cell and the ability to synthesize both RNA and proteins. Thus, 5-fluoro-2'-deoxyuridine (FUdR),[109] aminopterin, and 5-iodo-2'-deoxyuridine (IUDR)[110] blocked cellular DNA synthesis, but not interferon production. Mitomycin, which inhibits transcription, blocked interferon production.[111,112] Cogniaux-LeClerc[113] showed that ultraviolet irradiation of cells prior to induction by Newcastle disease virus resulted in inactivation of the cells' capacity to

produce interferon according to "one hit" kinetics. The authors concluded that damage to a single labile site located on DNA in the cell could block induction.

Similarly, Dactinomycin and trichlororibofuranosylbenzimidazole, both inhibitors of RNA synthesis, inhibit interferon production.[111,112,114] Inhibition of cellular protein synthesis by puromycin or p-fluorophenylalanine also inhibited production of functional interferon.[111,112,115]

Wagner and Huang[115] carried out a series of elegant experiments designed to study the effects of Dactinomycin and puromycin on NDV-induced interferon synthesis in Krebs-2 cells. They induced cells pretreated with puromycin to reversibly block protein synthesis. After an incubation period, the cells were exposed to Dactinomycin to irreversibly inhibit RNA synthesis and then washed to allow protein synthesis to commence. The interferon which was then produced was considered to have been synthesized from template RNA synthesized during the induction in the presence of puromycin. Thus, they indirectly demonstrated the rate of synthesis of RNA, presumed by the authors to be interferon-specific messenger RNA, necessary for interferon synthesis.

From this collection of data, one may conclude that production of interferon following induction by virus is a cell-directed function requiring synthesis of RNA and protein. However, one cannot conclude that this interferon is, in fact, newly synthesized following induction. The possibility that interferon may exist as an inactive precursor molecule within the cell must be considered. In this case, the requirement for new RNA and protein could be for synthesis of an activating enzyme or some other protein required for the activation event. A further analysis of this possibility and the evidence for its existence will be considered in conjunction with the discussion of interferon induction by complexed polynucleotides.

POLYNUCLEOTIDE INTERFERON INDUCERS. DISCOVERY AND CONFIRMATION OF ACTIVITY

Isaacs and his co-workers[116] suggested that "foreign" polynucleotides were active in inducing interferon in cell cultures. Moreover, these investigators claimed that chemical modification of homologous or "foreign" nucleic acids made these inducers more effective. The nucleic acids utilized in their studies were single-stranded and many workers, our laboratory among them, found it difficult to repeat these findings. Indeed, the English workers found it difficult to repeat their own work and finally abandoned the concept, favoring the idea that polysaccharides were the actual interferon inducers.[117] This concept gained favor when Kleinschmidt and his associates found that a preparation isolated from *Penicillium stoloniferum* induced interferon, and stated that their preparation was a polysaccharide.[118]

After our initial efforts to purify interferon, it was apparent to us that even if one were able to obtain ultimately pure material, the yields, criteria of ultimate purity, and the amounts needed were such that the use of exogenous interferon in human antiviral therapy did not predict a happy future. Moreover, the state of the art of mass cell culture was such that it did not add to any feeling of optimism concerning practical large-scale production of interferon, especially from safe human cell sources. Many investigators, Ho and Postic[119] among them, shared this feeling of pessimism. It should be stated, however, that many investigators did not and still do not share this point of view.[120]

With the abandonment of the concept of the use of exogenous interferon, it seemed appropriate to us to investigate further the findings of Shope[121] that an extract prepared from *Penicillium funiculosum* had antiviral activity and induced interferon. Lewis et al.,[122] working at the Merck & Co. laboratories, had obtained partially purified material from *Penicillium funiculosum* which was active in protection of mice against Columbia SK virus infection. Rytel et al.[123] later demonstrated that some substance in extracts of *P. funiculosum* induced interferon in animals.

A second valid approach was a careful chemical dissection of the virions of various viruses and a logical third approach was the examination of the products of virus replication. Many publications were appearing at that time on the occurrence of various replicative forms of RNA viruses of both animal and bacterial hosts.[124]

The task of isolating the active interferon inducing factor from *P. funiculosum* was completed, and its properties were known to be that of a ribonuclease resistant RNA. Tests with

certain polynucleotides in our laboratory soon yielded data to show that single-stranded polynucleotides such as polyadenylic acid, polyuridylic acid, polyinosinic, and polycytidylic acids were not active as interferon inducers, but when hydrogen bonded double-stranded complexes were prepared from complementary polynucleotides, active interferon inducers were achieved.[125]

The active materials which had the capacity to induce resistance to viral infection in cell culture and to induce the production of serum interferon in rabbits were poly rI:rC (rI_n:rC_n) and poly rA:rU (rA_n:rU_n).* It is of interest to note that Braun and Nakano[126] reported that polyadenylic and polycytidylic acids stimulated the rate of antibody formation in immune competent cells in vitro.

The purified preparation (RNA) from *P. funiculosum* was shown to be a ribonucleic acid which had the following properties:[127]

1. It contained ribose, guanine, uracil, adenine, and cytidine. It also contained phosphorus.
2. It did not contain deoxyribose, polysaccharide, or protein.
3. The ultraviolet spectrum was typical of a nucleic acid with maximum at 257 mμ.
4. It was relatively resistant to ribonuclease, but the biological activity could be destroyed by high levels of ribonuclease at high temperature.
5. It displayed a thermal denaturation curve typical of double-stranded nucleic acids with a Tm (thermal transition mid-point) at 95°C in low ionic strength buffer.
6. The biological activity was destroyed by addition of formaldehyde during denaturation since it prevented re-annealing of the strands.
7. The substance displayed no hyperchromic shift on addition of formaldehyde at 35°C indicating a stable highly ordered secondary structure such as a double-stranded helix.

The inducer isolated from *P. funiculosum* and the synthetic polynucleotide inducers had physical, chemical, and biological properties in common as follows:

1. Both were double-stranded ribonucleic acids.
2. They were active in microgram amounts in inducing host resistance in cell culture, interferon in rabbits, and host resistance to viral infections in mice.

At this point, it became apparent that either we were fortunate in finding three non-specific interferon inducers or that double-stranded ribonucleic acids as a class were all potential interferon inducers. It was soon apparent that double-stranded ribonucleic acids of viral origin were all effective interferon inducers when the double-stranded ribonucleic acid from Reovirus Type 3 virions and the replicative form of an RNA bacteriophage MS2 were isolated and proved to be highly active as inducers in cell culture, rabbits, and in mouse protection tests. We then postulated that the occurrence of the double-stranded ribonucleic acid in *P. funiculosum* must have been due to the presence of a mycophage. Banks et al.[128] later confirmed this hypothesis by isolating a mycophage from *P. funiculosum cultures*. Prior to the investigations by Banks et al., Kleinschmidt and his associates[129] had isolated a mycophage from *P. stoloniferum*, the organism from which statolon had been prepared.

In the course of the work with the *P. funiculosum inducer*, MS2 coliphage, and reovirus, we had also carefully prepared and tested a number of single-stranded RNA preparations and compared the activity with double-stranded RNA preparations for interferon induction in rabbits. It was obvious from these data that only the double-stranded molecules were active in inducing interferon in the rabbits. Tested among the preparations were single- and double-stranded RNAs from diverse sources, such as synthetic polynucleotides, mycophages, bacteriophages, animal viruses, plant viruses, insect viruses, yeast, animal liver, etc. Double-stranded DNA from calf thymus, however, was not active.[130] It would appear from these investigations that only double-stranded ribonucleic acids would be predicted to be active inducers of interferon.

In the course of the work with interferon inducers, we employed three systems for testing. The efficacy of these substances was assayed as follows:

1. Primary rabbit kidney cell culture was

*Poly I:C has been referred to as poly rI:rC, rIn:rCn, rIn-rCn, poly rI: poly rC, and poly I: poly C. These designations all have been applied to the double-stranded complex formed between the two homopolynucleotides, polyriboinosinic acid and polyribocytidylic acid.

used to demonstrate and quanitiate the induction of host resistance to viral infection. Most effective substances were active in this system in nanogram amounts. Vesicular stomatitis virus was used as a challenge infection.

2. Induction of interferon in rabbits was used as an in vivo test for inducers. Effective inducers of interferon were active in this system at levels of 0.25 to 0.5 μg per rabbit when the substance was injected intravenously. The rabbits were bled at two hours after injection and the serum was titrated in RK13 cell cultures for interferon titer. RK13 is a stable rabbit kidney cell line which is relatively insensitive to induction by double-stranded RNA, but sensitive to interferon.

3. A mouse test was used to assay efficacy of test substances as inducers of host reistance to viral infection. Mice (10-12 ICR) were pre-treated intravenously with inducer 3 hours prior to intranasal infection with 30 LD_{50} of pneumonia virus of mice (PVM). The mice were observed for death for 14 days.

In the paper by Field et al.,[125] a number of criteria were applied to the proof of requirement for double-stranding of complementary polynucleotides in order to induce host resistance in in vivo and in vitro and to induce interferon in rabbits as follows:

1. Effective complexing and formation of an active inducer were prevented by prior treatment of the poly C with formaldehyde.

2. Deamination of the amino groups of poly C with nitrous acid prior to complexing resulted in an inactive mixture.

3. Extensive shortening of the poly C chain by treatment with pancreatic ribonuclease prior to mixing with poly I prevented effective double-stranding and resulted in an inactive mixture.

4. Dissociation of the poly I:C complex at pH 9.5 markedly reduced biological activity.

It is interesting to note that although poly I:C and poly A:U had apparently equivalent activities in cell culture, poly I:C was vastly superior as an inducer of interferon in rabbits and of host resistance to viral infection in animals.

Confirmation of the activity of double-stranded nucleic acids as inducers of interferons was not long in coming. Dianzani et al.[131] not only confirmed the activity of poly I:C as an inducer but also made the interesting observation that DEAE-dextran increased the activity about 100-fold. Vilcek et al.[132] also found that single-stranded polynucleotides were inactive, whereas double-stranded polynucleotides were active inducers in cell culture. Perhaps the most significant confirmations of the phenomenon came from two laboratories. The first one was the work of Colby and Chamberlain.[133] Working in a chick embryo cell culture (Sindbis virus) system, these investigators, utilizing DEAE-dextran as an activator, made the following observations:

1. The double-stranded polynucleotide poly I:C is an extremely active inducer of resistance to Sindbis virus infection in chick embryo cells. The addition of DEAE-dextran remarkably increases the uptake of inducer by the cells and increases the reduction of virus titer in striking fashion.

2. Only double-stranded polyribonucleotides were active. All the carbohydrate residues must be ribose.

3. The interferon induction by double-stranded polyribonucleotides is acting with much greater specificity than other polyanionic substances, especially in the quantitative sense. The activity approaches a magnitude of 10^5 times higher, suggesting that the activity is in some way mimicking the natural induction process triggered by a replicating virus.

Falcoff and Perez-Bercoff[134] examined the optimal conditions for induction of interferon by poly I:C in two human cell systems, lymphocytes and cells derived from amniotic membrane. Their conclusions may be briefly listed as follows:

1. The mixing curve of poly I and poly C was examined and maximum activity of interferon induction was found at the point of greatest hypochromicity, where poly I and poly C were mixed in equimolar proportions.

2. DEAE-dextran increased the efficacy of poly I:C 100-fold.

3. Dactinomycin pretreatment of the cells reduced production of interferon indicating indirectly the requirement for DNA dependent protein synthesis.

4. The data were consistent with the hypothesis that the double-stranded configuration of the RNA molecule is required for the induction of interferon.

In addition to our own demonstration that

double-stranded ribonucleic acids from natural sources were potent inducers of interferon as well as inducers of host resistance in cell culture and in mice, two papers can be cited as confirming these observations. Falcoff and Falcoff[102] prepared the total tritium-labeled RNA from mengo virus infected L cells in culture. The mixture was fractionated in a sucrose density gradient. A fraction was found which was resistant to ribonuclease and formaldehyde. This fraction was an interferon inducer in mice and proved to be double-stranded. Single-stranded RNAs isolated from this system were not inducers of interferon in mice. In a recent publication, Inglot[104] isolated large amounts of interferon-inducing double-stranded RNA from Sindbis virus infected chick embryo cell suspensions. Nucleic acids extracted from the uninfected cells or from extracellular virus did not induce interferon. It may be the opinion of some molecular biologists that the isolation of double-stranded RNA from virus infected cells is an artifact of the fractionation procedure, but recent work by Stollar and Stollar,[105] employing immunofluorescent techniques, demonstrated that double-stranded RNA does exist as such in infected cells.

In Vitro Properties of Poly I:C

Poly I:C is capable of induction of resistance to viral infection in a wide variety of cells in culture.[135] It is apparent that various primary cell lines vary in their capacity to respond to induction by poly I:C from the very sensitive cells which respond to nanogram amounts to those which will not respond at all, such as WI-38, a human diploid lung cell. On the other hand, another human diploid cell (HFL–1B) was susceptible to induction. Similarly, certain viruses were more resistant to poly I:C than others. There was no correlation as to type (RNA or DNA) and variation existed within a group, such as the rhinoviruses. The observations by Falcoff and Perez-Bercoff[134] that poly I:C was active in human cells and by Field et al.[135] that the polynucleotide was active against rhinoviruses in human cells were especially significant for the possible use of the agent in human medicine against the "common cold" complex of viruses.

In Vivo Efficacy of Poly I:C Against Viral Infections in Animal Models

In the evaluation of any agent against virus infections, it is important to have readily available animal models which will yield some sort of prediction that ultimate success may be attainable in human and animal virus disease. The effective amount of drug as compared to the acute toxicity should yield data indicating the ratio of toxic level to protective activity, i.e., the prophylactic or therapeutic index. Several useful animal models were utilized by Nemes et al.,[136] to evaluate the potential of poly I:C.

The first system was the pneumonia virus infection of mice (PVM) which yielded an evaluation of poly I:C against a respiratory virus when the drug and virus were both given by the nasal route. The pneumonia virus of mice is lethal for the host. When the drug was given 3 hours prior to infection with 30 LD_{50} of virus, the prophylactic protection afforded by 1 μg of poly I:C was excellent. It has been our general experience that poly I:C will yield at least 40 to 60% protection against PVM at this level. Since it has not been physically possible to prepare a high enough concentration of poly I:C to assess toxicity by the nasal route, it has not been possible to calculate a nasal-nasal prophylactic index. However, if one assumes that the average intraperitoneal LD_{50} for the ICR 10-12 g mouse to be about 35 mg/kg then one may calculate the prophylactic index to be about 350. Such an evaluation yields a very conservative index since poly I:C is relatively non-toxic to animals by the intranasal route. The relationship of dose of virus to prophylactic efficacy was determined. The mice were given 16 μg of poly I:C intranasally 3 hours prior to infection. Virtually complete protection was achieved even against 10,000 LD_{50} of virus.

The prophylactic efficacy of poly I:C given intranasally three hours prior to nasal infection of mice had been well established, but no data were available with regard to duration of host resistance and, as yet, no therapeutic effect in this system had been demonstrated. Such experiments were performed and it was shown that mice were protected for a period of six days with a sharp drop at seven days. The therapeutic protection was fully effective for two days, somewhat effective for three days, with extinction achieved at four days. Mice may be infected with Columbia SK virus by the intranasal and subcutaneous routes. Data were also obtained indicating good protection of mice by poly I:C given by the intranasal and intraperitoneal routes against

Columbia SK virus given by the two routes of infection. These findings give support to the idea that the resistance is not strictly a local phenomenon, but is spread to different organs as would be expected if the resistance was the result of the induction of interferon.

PVM and Columbia SK are RNA viruses. A test of protective efficacy was performed with vaccinia, a DNA virus, and poly I:C was shown to exert a reasonable prophylactic effect given by two routes (intraperitoneal and subcutaneous) against an intravenous challenge of virus.

Other Animal Model Systems

The ready availability of poly I:C has enabled many investigators to test the efficacy of the polynucleotides in a variety of virus-host systems. Only a few examples will be cited here.

Park and Baron,[137] working with herpetic keratoconjunctivitis in rabbits, were able to demonstrate not only a striking prophylactic effect but also therapy several days after infection. These data are encouraging, since herpes simplex virus appeared to be quite resistant in cell cultures.[135] Whether such data can be translated to man remains to be seen. Hamilton et al.[138] demonstrated the activity of poly I:C in three systems; the protection of HEp 2 cells, as well as mice, against herpes simplex virus infections, and the protection of rabbits against herpes simplex-induced keratoconjunctivitis. These authors also confirmed the necessity of the double-stranded polynucleotide and inactivity of the single-stranded polynucleotides in achieving these results.

Richmond and Hamilton[139] induced resistance to foot-and-mouth disease in mice with poly I:C. Both prophylactic and therapeutic responses were observed. Single-stranded polynucleotides were not effective in this system.

Two papers on the antiviral efficacy of poly I:C are of special interest. The first, by Janis and Habel,[140] deals with the activity of poly I:C against intramuscular rabies virus infection in rabbits. It was demonstrated first that an intravenous dose of poly I:C given six times protected rabbits against lethal doses of mouse brain passaged fixed rabies virus. Even greater protection was afforded by direct injection of poly I:C into the site of virus challenge. The authors obtained evidence of rapid production of interferon at the site of intramuscular injection. Fenje and Postic,[141] also working with rabbits, infected the animals with virulent rabies virus obtained from an infected fox at a level which was lethal to all untreated animals. Excellent protection was obtained with a single intravenous dose of poly I:C given from 24 hours prior to infection to 6 hours post-infection. They attributed the protective effect to the induction of interferon. Not only did the treated animals survive the infection but also antibody levels developed in the serum, so that the animals survived a second challenge of virulent rabies virus when challenged 35 days after the original infection. Fenje and Postic suggest that the rabbit-rabies system may be a useful model in the treatment of rabies virus infection in man. Worthington and Baron[142] have recently published an interesting paper in which they showed that mice treated with immunosuppressive agents, such as cyclophosphamide, azathioprine, and 6-mercaptopurine, can be protected against a neurovaccinia infection, by poly I:C, even after the virus infection has spread to the point of development of tail lesions. The authors obtained a significant delay in lethal effects of the virus and a significant decrease in overall mortality. Apparently, immunosuppressive agents do not suppress interferon induction by poly I:C in the animal host.

In summary, many animal models of viral infections have yielded to treatment with poly I:C, demonstrating both prophylactic and therapeutic effects.

In addition to the induction of host resistance to viral infection in mice with poly I:C, Nemes et al.[143] have demonstrated that double-stranded replicative-form RNA, obtained from MU-9 coliphage infected *E. coli* cells, has much the same properties in prophylactic and therapeutic protection of mice against pneumonia, Columbia SK, Sendai, and vaccinia viruses.

There is no point in a repetitive recitation of all the work done on animal models with poly I:C. Many models have yielded favorable results, so that further effort in the evaluation of polynucleotides for use in human and animal medicine appears to be warranted.

Induction of Interferon in Human Subjects by Poly I:C

Prior to consideration of poly I:C as a candidate for human trial, the Department of Safety Assessment of the Merck Institute for

Therapeutic Research conducted extensive toxicologic investigations of the material and concluded that it was reasonably safe for cautious clinical investigation.[171] Assessment for safety was performed in mice, rats, dogs, monkeys, and hamsters. The dose levels, number of doses, and routes of administration were studied extensively. Detailed observations were made of physical signs, hematology, blood chemistry, gross pathology, and histopathology. Toxic effects were recorded which were not evident in the animals employed for demonstration of antiviral efficacy. Of the species tested, the dog appeared to be most susceptible to toxic effects of repeated intravenous injections of poly I:C. Rats and mice appeared to be less susceptible to toxic manifestations and the monkey the least susceptible. None of the animals tested by the respiratory route exhibited any significant toxic effects. The latter observations are most important from the viewpoint of application of poly I:C to potential use in man for the control of the respiratory infection. For further references to the toxicology of poly I:C, the reader is referred to Philips et al.[144] and Phillips et al.[145]

As an adjunct to the gross safety assessment, poly I:C was carried as a component of the medium in continuous passage of a human diploid lung cell strain in culture.[168] The presence of the polynucleotide did not cause any significant alteration of the growth rate, plating efficiency, and cell morphology, and did not bring about any delay or acceleration in the occurrence of senescence in the cultures. The karyotype of the cells was not altered, there were no chromosome breaks, and neoplastic transformation did not occur. Similar results were obtained with the double-stranded replicative-form RNA of the mutant coliphage Mu 9 (an amber protein coat mutant of MS 2 coliphage). Poly I:C was not carcinogenic when tested in newborn hamsters and it did not accelerate the growth of the human tumor cell HEp-2 in the hamster cheek pouch.[146,147]

Several publications have referred to the prophylactic and therapeutic effect of poly I:C against virus induced cell transformation[148] and transplant tumors[149] in animal species. The mechanism of action of poly I:C in this respect is, as yet, unknown. Cautious trials in man were initiated to test for activity against cancer under clinical conditions suitable for that purpose.[150] Samples of blood were taken prior to initiation of treatment with poly I:C and at various intervals following treatment for the purpose of quantitative assay of interferon induction. All treatment with poly I:C was by the intravenous route. The patients were all in advanced stages of cancer.

Twenty patients were treated with 2 μg to 4000 μg of poly I:C per kilogram of body weight. Fourteen of the patients responded with production of an antiviral substance in the serum which was identified as interferon by the usual criteria such as: trypsin sensitivity, pH stability, host species specificity, non-sedimentability at 82,000 XG for one hour, and broad virus spectrum. Nine of the patients had no pre-titer, while five responded from a pre-titer of four units. The titers ranged from 4 to 32. Interferon appeared as early as 2 hours after treatment with poly I:C and persisted in some of the patients for 72 hours or more. The highest titer appeared in most cases 12 to 48 hours after administration of poly I:C. Within the dose range and in the limited number of patients tested, there appeared to be no dose-related magnitude and duration of response.

Re-induction was possible after an adequate time period and the same patient was capable of being re-induced many times. Definite signs of refractoriness were noted much the same as has been noted in small animal species.

With regard to clinical observation, the most consistent response to intravenous injections of poly I:C was a febrile reaction. The rise in temperature ranged from 0 to 7°F and usually reached a peak at 6 to 15 hours post treatment. The febrile response was usually accompanied by interferon induction, although each response could occur independently. There appeared to be no correlation with dosage. Laboratory tests showed no disturbance of bone marrow, liver, or kidney functions. No disturbances of clotting factors were found, and no limiting toxic effects were noted in clinical symptoms.

Tests were performed to detect an immune response to poly I:C. No detectable antibody titers to poly I:C or heat denatured DNA were found in serum samples from 19 patients tested prior to and post treatment. Complement fixation was employed to detect such antibodies with standardized negative and positive rabbit sera as controls. These results, of course, must be interpreted with some

caution because of the possible lack of immune competence in these patients.

It was surprising to find the lack of clinical reactions in man, other than the febrile response, in view of the toxic manifestations obtained in other animal species. Further assessment of safety for man must await further cautious trials in normal humans. It is also imperative to evaluate poly I:C in man as a prophylactic and therapeutic agent against virus infections by the respiratory route.

PHYSICOCHEMICAL STUDIES OF POLY I:C

Effect of Polyamines on the Induction of Interferon and Host Resistance by Poly I:C

Cohen and Raina[151] have discussed the existence of polyamines in living cells and the possibility that they may have an important role in the synthesis and activity of nucleic acids. The authors stated that certain polyamines increase the thermal stability of deoxyribonucleic acid. Dianzani et al.,[131] reported the enhancement of the induction by poly I:C of interferon in cell culture by DEAE-dextran and later these investigators reported an enhancement of the protective effect of poly I:C against Columbia SK virus infection in mice.[152] The effect of DEAE-dextran in cell culture has now been confirmed by many laboratories. The in vivo effect, however, has not been confirmed. The use of DEAE-dextran in human medicine would be of questionable value because large polycations form aggregates with the polynucleotides and would probably increase the potential antigenic qualities of the nucleic acids. Rosenquist,[153] working with poly I:C in calves, was not able to demonstrate enhancement of interferon titers nor an extension of interferon residence time in the blood. The well demonstrated effect of DEAE-dextran on the enhancement of poly I:C activity in cell culture may well turn out to be a useful tool in the study of the mechanism of action of double-stranded polynucleotides in the induction of interferon.

Billiau et al.[154] reported that certain polyamines enhance the in vitro effects of synthetic polynucleotides. In particular, it was demonstrated that neomycin and streptomycin were effective in enhancing the activity of poly I:C in cell culture. Lampson[155] and his co-workers undertook a detailed study of this phenomenon from the following aspects: influence on the induction of interferon by poly I:C and induction of resistance to viral infections in vitro and in vivo; cell uptake of polynucleotide; thermal stability; toxicity for mice; and sensitivity of polynucleotide-polyamine mixtures to ribonuclease. The results of these studies may be summarized as follows; that neomycin:

1. added to poly I:C enhanced the in vitro activity in rabbit cell cultures about 17-fold confirming the observation of Billiau et al. This phenomenon was cell dependent since there was no enhancement of poly I:C activity in RK 13 and grivet monkey kidney cells. There was some evidence of suppression of activity in these cell cultures;
2. added to poly I:C or poly A:U, it did not enhance the induction of interferon in rabbits;
3. did not activate single-stranded polynucleotides as inducers of interferon in rabbits;
4. added to poly I:C did not enhance the activity against pneumonia virus nor against Sendai virus in mice;
5. suppressed rather than increased the uptake of poly I:C by rabbit kidney cells in culture;
6. did increase the thermal stability of poly I:C when the ratio of polyamine to polynucleotide was about 6 to 9:1;
7. did not affect the acute toxicity of poly I:C for mice.
8. It did inhibit the degradation of poly I:C by pancreatic ribonuclease. On the other hand, it did not inhibit the degradation of poly C, a single-stranded polynucleotide by ribonuclease.

In summary, the enhancement of interferon induction and host resistance to viral infection by poly I:C-polycation mixtures remains an interesting in vitro phenomenon. It is undoubtedly useful as a tool in those cell cultures where significant enhancement of activity is achieved as in the studies of Colby and Chamberlain[133] in chick embryo cells. It has, as yet, no significant usefulness in the in vivo action of polynucleotides as inducers of host resistance to viral infections.

Relationship of Molecular Size of Poly I:C to Induction of Interferon and Host Resistance

It has been a general experience in our labora-

tories that samples of homopolynucleotides purchased from various commercial sources when complexed to form double-stranded polymers exhibit considerable variation in capacity to protect mice against viral infections. This variation in activity led us to speculate that the molecular size of the complex could be a factor and such studies were made by Lampson et al.[156]

The polyinosinic acid and polycytidylic acid were prepared in the Merck Process Development Laboratories with highly purified polynucleotide phosphorylase prepared from *Micrococcus lysodeikticus*. Special precautions were taken to ensure highest purity and especially to preclude microbial contamination. The products of this synthesis yielded the classical physicochemical characteristics and, in addition, the individual homopolymers were not pyrogenic in rabbits. The complexed polynucleotides were prepared by mixing equimolar amounts as outlined by Field et al.[126]

It was found that progressive cleaving of the complexed poly I:C could be achieved by sonic radiation. The cleavage of the molecule could be followed by a simple determination of viscosity. As a further characterization, a sepharose elution pattern was performed on each preparation. When related to the parent compound, the effect of sonic radiation was discernible by relative viscosity, Tm (°C), hyperchromicity on melting, $E_{1\%}$ at 265 mμ and relative sensitivity to pancreatic ribonuclease. Molecular weights were calculated from the equation, $MW = 1.15 \times 10^3 \times S_{w,20}^{2.9}$ where $S_{w,20}$ is the sedimentation coefficient. This equation was developed by Studier[157] and was used based on the assumption that the conformation of poly I:C is similar to that of double-stranded DNA.

Three biological parameters were measured for each of the sonic-treated complexed polynucleotides as follows:

1. activity in inducing resistance to virus in cell culture (rabbit kidney cells-vesicular stomatitis virus).
2. induction of interferon in rabbits.
3. protection of mice by the intranasal route against intranasal infection with pneumonia virus.

Although the reduction in molecular weight was approximately 27-fold with respect to the parent compound, the complex with a molecular size of 2.8×10^5 still retained fairly good activity in cell culture and in rabbits. In the mouse protection assay there was a sharp drop in activity with a 2.8-fold reduction in molecular weight and a very significant drop in activity with a 6.5-fold reduction in molecular weight. Evidently the mouse assay was the most sensitive method of determining the effect of reduction in molecular size and within these experiments a molecular size of at least 4.2×10^6 or greater was required for maximum activity in the mouse-PVM system.

In order to assess the effect of molecular size on the cell culture and rabbit interferon systems with a greater degree of precision, fractions of sonic-treated poly I:C were taken from a sepharose column and tested in the two systems. The induction of interferon in the rabbit (at 2 μg per rabbit) occurred at a molecular weight of approximately 1.2×10^5 or greater, with the cell culture system falling off at or below that figure.

One can conclude that the capacity of poly I:C to induce interferon and host resistance to viral infections in cell culture and in mice is a function of molecular size. Under the conditions of the experiments, the Tm, hyperchromicity on melting, and ribonuclease sensitivity were also functions of molecular size. The shape of the ultraviolet spectrum and the $E_{1\%}$ were not significantly affected by molecular size as achieved by sonic treatment.

Influence of the Size of Individual Homopolymers on the Physical and Biological Characteristics of Complexed Poly I:C

Since molecular size of the poly I:C complex did affect the biological activity in three types of assays, it was logical to study the effects on the physical and biological properties of poly I:C complexed from homopolymers of various molecular sizes. Tytell et al.[169] made such a study. Various molecular sizes of poly C were prepared in three ways as follows: (1) by enzymatic synthesis; (2) by sonic degradation; (3) by ribonuclease degradation. Various sizes of poly I were achieved only by enzymatic synthesis since this polymer was resistant to both sonic degradation and digestion by pancreatic ribonuclease. In this work, once again, three assay systems were used:, (1) viral resistance in cell culture; (2) induction of interferon in the rabbit; (3) host resistance to pneumonia virus infection in the mouse.

The results of these investigations can be summarized as follows:

1. poly C can be degraded by sonic treatment to smaller fragments to limit of $S_{w,20} = 4.0$. The end-product has the same capacity to complex with poly I as the original polymer ($S_{w,20} = 9.2$). The viscosity of the new complex was significantly lower than the parent complex. The biological activities of the new complexes were not significantly different than the parent complex.

2. Treatment of poly C with ribonuclease for varying time periods significantly changed the capacity of poly C to complex with poly I resulting in significant shifts in ultraviolet absorbency and shifts in peak. The viscosity of the complexes decreased with increased degradation of the poly C. Hypochromicity on complexing fell off rapidly as poly C was progressively digested. The biological activity decreased significantly in the mouse protection test and somewhat more slowly in the cell culture protection test. It was significant that even with a hypochromicity of 7.1%, the complex still protected rabbit kidney cell cultures against virus infection at a level of 0.13 μg. As was expected, the complexes were relatively more ribonuclease sensitive as poly C was progressively degraded.

3. In an experiment to determine the importance of the relative molecular sizes of each of the components of the double-stranded complex, an experiment was designed to keep one homopolymer constant while varying the size of the other component. These complexes were tested in cell culture. With poly I constant at $S_{w,20} = 9.2$, poly C with only 70 nucleotide residues was still as active as a combination of poly I (9.2): poly C (13.2). With poly C constant at $S_{w,20} = 9.2$, the activity broke sharply with poly I between 240 and 550 nucleotide residues. Evidently the requirement for poly I of larger molecular size than 8.4×10^4 was established.

The translation of this experiment to protection in animal-virus models clearly demonstrated the requirement for relatively high molecular size of poly I.

These studies should be useful in establishing the importance of defining the components of poly I:C and the complex prior to designing experiments as to the efficacy of the drug in certain virus infections.

MECHANISM OF INTERFERON INDUCTION BY COMPLEXED POLYNUCLEOTIDES

The availability of a chemically defined, highly potent interferon inducer active in cell cultures has restimulated research into the mechanism of interferon induction. As previously discussed, detailed chemical and physical studies into the nature of the inducer molecule are well under way, so that a clearer definition of the factors influential in altering inducing capacity is now available. As a result of these studies, using complexed polynucleotides, direct design of efficient inducers will be possible and more insight into the nature of virus-associated interferon inducers may be obtained. These studies of the inducer molecule have been one phase of the study of mechanisms. Another phase must relate to the interaction between inducer molecule and the inducible cells, with the resultant production of interferon.

Cellular Uptake and Fate of the Inducer Molecule

Recent studies involving radiolabeled poly I:C have traced the interaction of this potent interferon inducer with inducible cells. Field et al.[158] demonstrated that during the first two hours of exposure of primary rabbit kidney cells to 10 μg poly I:C/ml, interferon production was initiated. Yet, during this exposure, only about 1% of the available inducer became cell-associated. Furthermore, the uptake of even this amount was not correlated with the capacity of poly I:C to induce interferon. The inactive single-stranded polynucleotides poly I and poly C, and even the precursor nucleotides inosine diphosphate and cytidine diphosphate became cell-associated at rates comparable to that of poly I:C.

Bausek and Merigan[159] further showed in similar studies that the binding of poly I:C to inducible human foreskin cells occurred at either 4°C or 37°C. A similar amount of poly I:C binding occurred in mouse L cells, although they were not inducible under the conditions tested. Again, uptake of inducer could not be related to capacity to induce interferon production.

The distribution of radiolabeled poly I:C within inducible cells has been analyzed by both cell fractionation and autoradiography. Following a two-hour exposure of primary rabbit kidney cells to poly I:C, C_{14} labeled in either complementary polynucleotide, half of the label became

associated with the cytoplasmic fraction and half the label became associated with purified nuclei (Field – unpublished observations). By autoradiographic analysis, using the electron microscope, Prose et al.[160] demonstrated that within one-half hour of exposure to tritiated poly I:C, primary rabbit kidney cells carried label in the nucleus, nucleolus, and cytoplasm. By two hours, label was concentrated primarily over the nucleolar region. Subsequently, label became dispersed over the cell. These data supported an earlier observation by Bausek and Merigan[159] that poly I:C taken up by the cell is degraded to precursors used for synthesis of cellular RNA. Thus, from studies of the fate of radiolabeled poly I:C, no specific interaction of inducer with an interferon-associated receptor has been revealed, and no critical information concerning the initiation of interferon production was obtained. However, these studies have emphasized that minute quantities of double-stranded RNA were capable of interferon induction.

Production of Interferon Following Induction by Complexed Polynucleotides

The nature of polynucleotide-induced interferon production and its potential significance as a model for virus-induced interferon production have also been intensively studied recently. As in so many studies of mechanism, metabolic inhibitors have been used extensively. The results of these studies have been controversial and have revealed both the pitfalls of relying on indirect evidence and the complexity involved in the mechanism of interferon production.

Field et al.[135] demonstrated that pretreatment of primary rabbit kidney cell or rabbit spleen cell cultures with Dactinomycin prior to exposure to poly I:C resulted in inhibition of interferon production. Furthermore, these same authors showed that following the initiation of interferon production, continuous exposure of cells to cycloheximide or puromycin greatly suppressed production of interferon. Thus, it appeared that poly I:C did stimulate the *de novo* synthesis of protein associated with interferon production in these cell cultures. A totally different result was obtained by Youngner and Hallum[161] in studies of poly I:C-induced mouse interferon. Under the conditions of their tests, cycloheximide treatment of animals enhanced the production of serum interferon. Also, Vilcek et al.[162] observed a lack of inhibition and even an enhancement of poly I:C induced interferon production by Dactinomycin or puromycin. These authors suggested that poly I:C induced interferon may be produced from inactive precursors rather than being newly synthesized at the time of induction.

This apparent inconsistency of results has been studied more fully now in several animal cell systems with the emergence of a new awareness of the complexity of this induction process. Further analysis of the effects of metabolic inhibitors on rabbit kidney cell interferon production led Vilcek[163] to postulate that after initiation of interferon production, a regulator protein is synthesized. This second protein shuts off the production of interferon at about four hours after the initiation of interferon production. While inhibitors of protein synthesis, such as puromycin and cycloheximide and the inhibitor of RNA synthesis, Dactinomycin, inhibit the synthesis of interferon, they also inhibit production of the regulator protein. The particular effect, enhancement versus suppression of interferon production, could be explained by the relative suppression of interferon formation versus formation of the regulator protein.

Tan et al.[164] have further elaborated on the mechanism of control of interferon production induced by poly I:C and ultraviolet irradiated Newcastle disease virus. Again, depending on the test condition, cycloheximide and Dactinomycin suppressed or enhanced the in vitro production of interferon. The regulation of interferon production was similar, regardless of whether the inducer was complexed polynucleotide or virus. The proposed mechanism suggested that (1) the inducer triggers the synthesis of interferon messenger RNA by the cell, (2) this messenger RNA is translated into newly synthesized protein (probably inactive or active interferon), (3) the protein synthesized in (2) initiates transcription of messenger RNA which is, (4) translated into a regulator protein. The regulator protein inhibits production of interferon. According to this hypothesis, early inhibition of messenger RNA synthesis would prevent interferon formation, whereas late inhibition of messenger RNA synthesis would primarily inhibit synthesis of regulator protein, enhancing interferon production. Such an effect was observed when Dactinomycin was employed as a metabolic inhibitor. Early inhibition of protein synthesis

blocks interferon production, but later blockade by cycloheximide enhances interferon production by suppression of production of the regulator protein. Tan et al. emphasize that stimulation of interferon production by double-stranded RNAs, like poly I:C, and by viruses may involve this common mechanism. The differences observed between viruses and double-stranded RNAs as inducers may merely represent the steps necessary for virus-infected cells to synthesize the double-stranded RNA.

Ho and Ke[165] have chosen to study the in vitro production of interferon in tissues of rabbits obtained within minutes after intravenous injection of poly I:C. Such an approach has allowed analysis of the contribution of different tissues to the interferon observed in animals, and analysis of the role of macromolecular synthesis in interferon production. Lung, liver, and spleen tissue slices were the main interferon producers. By use of metabolic inhibitors, these authors concluded that stimulation of interferon production required new protein synthesis. Interferon production did not represent simply the release of a performed interferon molecule. They suggest that, instead, the induction process might be a two-step process involving synthesis of a "preinterferon" followed by conversion into active interferon. Ke and Ho[166] have further shown that whereas cycloheximide inhibited production of interferon in liver and spleen slices induced in vivo either by poly I:C or Newcastle disease virus, similar treatment of kidney and lung slices could enhance interferon production. By using both puromycin and cycloheximide, interferon production was prevented. These data were consistent with the hypothesis proposed by Tan et al.[164] and would reflect preferential inhibition of either the direct interferon synthesis or synthesis of the postulated regulator protein.

At this early stage in the analysis of the mechanism of induction of interferon, only a few conclusions can be made. It now appears that complexed polynucleotides do induce the production of interferon in a process which requires *de novo* synthesis of RNA and protein. This requirement may reflect the *de novo* synthesis of active or precursor interferon or some other protein necessary for production and release of the interferon. Evidence now available also indicates that complexed polynucleotides and viruses induce interferon by similar or identical mechanisms. Differences in the two induction patterns could reflect additional steps involved in production of the active inducer molecules in virus-infected cells. Finally, the studies, to date, have indicated that the effects of metabolic inhibitors may vary greatly depending upon the relative time of induction and treatment with inhibitor, the concentration of inhibitor used, and the cell population used for induction studies. These varied effects may reflect the complex interferon production regulation system triggered by interferon inducers.

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ADDENDUM

The selection of literature used for this review was completed in March 1971. Since then, several articles dealing with interferon research have appeared in the literature. In our opinion, the observations of Pitha, J. and Pitha, P.M., Antiviral resistance by the polyinosinic acid – Poly (1-vinylcytosine) complex, *Science*, 172, 1146, 1971, warrant further discussion.

Pitha and Pitha investigated the antiviral activities of several analogs of poly I:C. Unexpectedly, the complex of poly I with poly (1-vinylcytosine) containing about 7% uracil was as active as poly I:C as an inducer of resistance to vesicular stomatitis virus infection in human skin fibroblast cells in vitro. However, poly I:C induced about ten times more intracellular interferon than did poly I:poly (1-vinylcytosine). Consequently, the antiviral activity of the later complex may not have all resulted from the interferon mechanism.

If this observation of antiviral activity by poly I:poly (1-vinylcytosine) is due to its capacity as an interferon inducer, then some reevaluation of the requirement of complexed polynucleotides for induction would be in order. Poly (1-vinylcytosine) differs from poly C by lacking any sugar phosphate backbone and by carrying no charge. When poly (1-vinylcytosine) is complexed with poly I, four cytosine residues occur for each inosine and the complex forms large aggregates. Thus the complex is markedly different from poly I:C. A comparison of their chemical and physical similarities and differences should be helpful in determining the characteristics of interferon inducers.