Studies on the Mechanism of Interferon Action

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ABSTRACT Interferon does not inactivate viruses or viral RNA. Virus growth is inhibited in interferon-treated cells, but apart from conferring resistance to virus growth, no other effect of interferon on cells has been definitely shown to take place. Interferon binds to cells even in the cold, but a period of incubation at 37°C is required for development of antiviral activity. Cytoplasmic uptake of interferon has not been unequivocally demonstrated. Studies with antimetabolites indicate that the antiviral action of interferon requires host RNA and protein synthesis. Experiments with 2-mercaptop-1(β-4-pyridethyl) benzimidazole (MPB) suggest that an additional step is required between the binding and the synthesis of macromolecules. Interferon does not affect the adsorption, penetration, or uncoating of RNA or DNA viruses, but viral RNA synthesis is inhibited in cells infected with RNA viruses. The main action of interferon appears to be the inhibition of the translation of virus genetic information probably by inhibiting the initiation of virus protein synthesis.

Interferon does not directly inactivate virus particles. It blocks an intracellular step involved in virus replication, probably virus-directed protein synthesis. Interferon-treated cells seem to develop this resistance to viruses through an active process involving cellular RNA and protein synthesis. The mechanism of action of interferon therefore involves the following two distinct problems: the changes which take place in an interferon-treated cell that lead to the development of resistance to virus growth, and how events in the virus growth cycle are modified by interferon treatment.

The noteworthy properties of interferon should be accounted for by observations on its mechanism of action. These properties include inhibition of DNA and RNA virus replication, a remarkable selectivity of interferon action which results in inhibition of virus replication with as yet no detectable effect on cell metabolism, the variation in sensitivity of viruses to interferon, its species specificity, and its remarkable potency. What has been discovered so far about interferon action does provide data which help to explain some of these properties. At the end of this report I will try to show, at least provisionally, how current ideas on interferon action may explain some of its properties.

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LACK OF EFFECT ON UNINFECTED CELLS

The only unequivocal way so far discovered of demonstrating an effect of interferon is by showing that interferon-treated cells are less able to support viral growth than untreated cells. A few other responses of the cell to interferon have been reported, but these observations were made with crude or only partially purified preparations. Obviously, any alterations in cellular function observed in interferon-treated cells may be of relevance to the mechanism by which the antiviral state is expressed, but despite the importance of this approach, there have been few salient studies on the effect of interferon on cellular metabolic activity, and all of these studies have had negative results. The reason for this is that meaningful results can only be obtained in this case with highly purified interferons. Such work has failed to confirm results obtained in earlier experiments in which crude interferon was used. Thus, reports on an inhibition of cellular RNA synthesis were not confirmed when highly purified interferon was used (1), and effects on glucose metabolism (2) were not observed with purified material (3). Similarly, purified interferon had no effect on cell division (4), an action which had been reported earlier. Furthermore, cells have been maintained in culture and passaged continuously exposed to interferon for prolonged periods without apparent deleterious effects (5, 6).

It is clear that interferon has no gross effect on cellular RNA and protein synthesis. Because of the problem of impurities, it is the negative results that are significant, since these were obtained under conditions where interferon was exerting a strong antiviral effect. Thus, if cellular RNA and protein synthesis are involved in the development of resistance, these are in the synthesis of a specific cell protein or RNA rather than in the over-all amount of RNA and protein made.

THE INTERACTION BETWEEN INTERFERON AND THE CELL

Small amounts of added interferon bind to the cell surface as a necessary prelude to the development of antiviral activity. While this step is necessary, it is not sufficient for the development of antiviral activity which still may be blocked by an inhibitor of cellular RNA synthesis.

Chick cells incubated with interferon at 1°C for several hours only develop antiviral activity after a subsequent incubation at 37°C following removal of interferon by repeated washings (Table I). Addition of actinomycin D immediately after the incubation at 1°C, however, blocked the development of antiviral activity. Therefore a period of incubation at 37°C is required for appearance of antiviral activity (7-10). An interaction between interferon and the cell does, however, occur in the cold. When interferon was incubated at 1°C with cells which were then washed many times and incubated at 37°C,
the cells were found to develop a degree of antiviral activity proportional to the concentration of interferon and the incubation time in the cold; however, a maximal response for any concentration was reached with a 10–20 min incubation. These results suggested that an equilibrium reaction involving interferon and a cell binding site might be taking place at 1°C (Fig. 1).

In order to test whether this reaction involved the binding of interferon to a superficial site, the cells were sequentially incubated with interferon at 1°C, washed, and treated at 1°C with a concentration of trypsin sufficient to destroy any interferon accessible to the enzyme. In these experiments antiviral activity failed to develop (Table II). This result indicated that interferon binds rapidly and firmly to superficial sites on cells and that this binding is necessary for development of antiviral activity (10, 11). The binding site was not destroyed by treatment of cells with trypsin (Table II) or with phospholipase C (R. M. Friedman, unpublished observation).

It might be logically supposed that the next step would involve uptake of interferon, but there is no convincing experimental evidence that entry of interferon into the cell is also necessary for development of antiviral activity. In early studies crude interferon seemed to be slowly removed from culture fluids by cells (8, 12–14). In later experiments with purified chick and mouse interferons, 80% of the interferon initially incubated with cells was not detected in culture fluids after 5 hr (15), but the disappearance of interferon from the above described culture fluids was not equivalent to its entry into cells since purified interferons adsorb nonspecifically to various surfaces. Also Buckler et al. (16) showed that less than 7% of the interferon incubated with cells was removed from the culture fluids in the course of the establishment of antiviral activity. Therefore, considerable uptake of interferon was not necessary for its antiviral action to develop. However, poly-L-ornithine

| Treatment          | Initial incubation | Add. 7 hr at 37°C |
|--------------------|--------------------|-------------------|
| None, 37°C         | 17                 | 20                |
| Interferon, 37°C   | 1.8                | 1.2               |
| None, 1°C (+Act. D)| 13                 | 14 (15)           |
| Interferon, 1°C (+Act. D)| 10 | 2.0 (14) |

* Chick cells were incubated with 3 units of interferon at 37°C or 1°C. After 4 hr the cells were washed and immediately infected with Semliki Forest virus or incubated an additional 7 hr at 37°C with or without 2 µg/ml of actinomycin D (Act. D) and then infected. After 8 hr of infection the infected cultures were frozen and thawed, and the fluids were assayed for virus yields. Figures in parenthesis are the results in experiments performed in cells treated with Act. D.
which generally increases adsorption to and entry into cells of macromolecules was reported to increase interferon action significantly (17). This latter finding suggests that some interferon may enter cells and that this step is a second prerequisite, after adsorption, for interferon action.

Much information is available on subsequent steps which lead to the establishment of the antiviral state in interferon-treated cells. Unfortunately, al-

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![Figure 1](image_url)

**Figure 1.** Interferon–cell interaction at 1°C. Chick cells were incubated with various concentrations of interferon for the indicated periods of time, washed six times with cold medium, incubated at 37°C for 2 hr, and then infected with virus. The virus titer was assayed on chick monolayers by a plaque technique and is reported as a percentage of the titer on control cultures which had not received interferon treatment.

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**Table II**

| Treatment* | Virus titer (p.f.u. × 10^-6) |
|------------|-----------------------------|
| None       | 18                          |
| Interferon (1000 U/ml) | 3.0                        |
| Trypsin (3 mg/ml)   | 5.7                        |
| Trypsin after interferon | 5.2                      |
| Interferon after trypsin | 1.2                      |

* Chick cells were incubated with 1000 U/ml of interferon for 20 min at 1°C. After washing they were treated with 3 mg/ml of trypsin, followed by soybean trypsin inhibitor (6 mg/ml), and incubated at 37°C for 2 hr. The cells were then infected with SFV, and virus yields after 8 hr were assayed.

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most all of this data is based on findings employing potent antimetabolites (Table III), and the use of such drugs involves some difficulties in that their total effects on cells are not yet clearly understood. Nevertheless, these studies have helped to clarify some aspects of the interferon–cell interaction. Specifically, acquisition of resistance probably involves synthesis of RNA and protein, and at least one other active function by the host cell.

Taylor (18) found that actinomycin D, which inhibits DNA-dependent RNA synthesis prevented the development of resistance in response to inter-
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The reduction in single cycle yields of SFV in chick embryo cells treated with interferon was not seen if the cells were treated with actinomycin before or together with interferon. Moreover, not only was virus production greater than in cells treated with interferon alone, but viral RNA synthesis, which was markedly depressed by interferon, was restored to a near normal rate when actinomycin was added before interferon. Actinomycin added after interferon did not reverse well-established resistance. Thus, it is the development of resistance, and not its expression, that is sensitive to actinomycin. In fact, no inhibitor of established interferon action has as yet been discovered.

The findings with actinomycin have been repeatedly confirmed (19, 20), but

| TABLE III | ANTIMITABOLITE INHIBITORS OF INTERFERON ACTION |
|-----------|-----------------------------------------------|
| Site of action | References |
| RNA synthesis | |
| 1. Inhibitors | |
| Actinomycin D | (18, 19, 20) |
| 6-Mercaptopurine | (23) |
| 6-Azauidine | (22) |
| 2. Miscoding | |
| 5-Fluorouracil | (22) |
| Azacytidine | (R. M. Friedman, unpublished observation) |
| Protein synthesis | |
| 1. Inhibitors | |
| Puromycin | (19, 20, 24) |
| Cycloheximide | (23; R. M. Friedman, unpublished observation) |
| 2. Amino acid analogues | |
| Fluorophenylalanine | (25, 26) |
| DNA function | |
| 1. Cross-linker | |
| Mitomycin C | (23) |
| 2. Thymidine analogues | |
| Iododeoxyuridine | (23) |
| 5-Mercaptomethyl uracil | (23) |

the dose-response curve of actinomycin action on interferon follows an unusual pattern in that a jump from zero to complete inhibition occurs in a narrow drug concentration range (Fig. 2), and it is possible that the effect of actinomycin results from an action other than its suppression of DNA dependent RNA synthesis. Indeed, some of the biological effects of actinomycin D are not easily explained by a primary effect on RNA synthesis only (reviewed in reference 21). However, other inhibitors of RNA synthesis with different mechanisms of action have also been reported to block the antiviral action of interferon to some extent; 6-azauridine which inhibits RNA synthesis, and 5-fluorouracil which is incorporated into RNA thereby causing miscoding, both inhibit interferon (22). Two other RNA inhibitors, azacytidine (R. M.
Friedman, unpublished observation) and 6-mercaptopurine (23), had a similar effect. The results obtained with these drugs were, however, not as impressive as the findings with actinomycin.

Development of resistance was also impaired by an inhibitor of protein synthesis, puromycin (20, 24), and by the amino acid analogue, fluorophenylalanine (25, 26). As with actinomycin, the time of adding these inhibitors in relation to interferon was important. When puromycin was added for 4 hr, after a 4 hr treatment with interferon, little inhibition of interferon action was observed (24). When cells were exposed to interferon for 1 hr and then incubated for a further 4 hr in an interferon-free medium before challenge, an increase in resistance during this 4 hr period was observed. This was not seen, however, if puromycin was present during this latter period (24). In the same study, there was a recovery of resistance when cells, which had been incubated for a 5 hr period in the presence of interferon and puromycin, were maintained for a further 20 hr after washing out the puromycin before virus challenge. These results, taken together, suggest that a period of protein synthesis is required after exposure to interferon for resistance to develop.

Interesting results on the effect of another inhibitor of protein synthesis, cycloheximide, on development of resistance in mouse L cells were obtained by Dianzani et al. (26). Pretreatment of cells with interferon in the presence of this inhibitor had no effect on the development of resistance. In other cells, however, cycloheximide was effective in preventing the development of resistance (23) (F. Dianzani and R. M. Friedman, unpublished observations).

The effects of other metabolic inhibitors have been studied. Mitomycin C,
iododeoxyuridine, and 5-mercaptopurinomethyl uracil inhibited interferon action, while 5-fluoro-2-deoxyuridine did not (23). The latter drug inhibits DNA synthesis, while the others have an additional effect on the function of DNA, either by causing cross-linking of DNA molecules (mitomycin C) or by acting as thymidine analogues. These results indicate that functional DNA as well as RNA and protein synthesis are required for interferon action.

2-Mercapto-1 (β-4-pyridethyl) benzimidazole (MPB) has also been shown to inhibit interferon action (Fig. 3). The drug has no effect on cell RNA or protein synthesis in concentrations which inhibit interferon action. The inhibitory effect on interferon action was reversed by simply washing the cells. MPB has no effect on established interferon action or on the binding of interferon. The site of MPB action would appear to be on a step following interferon binding as evidenced by the following experiment (Table IV). Cells were incubated with interferon at 1°C for 10 min or 1 hr. They were then washed with cold medium, MPB was added, and the cells were warmed to 37°C for 4 hr. MPB treatment had no effect on interferon action if the incubation at 1°C was permitted to proceed for 1 hr, but its action was blocked by MPB addition after a 10 min incubation at 1°C (R. M. Friedman and I. Pastan, manuscript in preparation).
Evidence for a step following binding but before RNA and protein synthesis has not been previously reported, although an investigation of the mechanism of action of polypeptide hormones has revealed an analogous picture. The action of thyroid-stimulating hormone (TSH) previously bound to thyroid cells was inhibited by incubation with anti-TSH antiserum if the binding reaction had been allowed to proceed in the cold for 5 min. Little effect of the antiserum was seen following a 1 hr incubation (27).

MPB has also been shown to inhibit phospholipid synthesis. It is possible that the step in interferon action which is blocked by MPB involves phospholipids; therefore, among possible sites for this step may be penetration of cells by interferon, induction of alterations in membrane properties by interferon, or induction of a new protein by interferon. It is of interest that the uptake of nucleosides in many cell lines (28), and the induction of tyrosine transaminase in rat cells (G. Tomkins, personal communication) and of aryl hydro-

| Treatment                | SFV yield (p.f.u./0.2 ml) |
|--------------------------|---------------------------|
|                          | 10 min incubation | 1 hr incubation |
| None for 10 min or 1 hr  |                          |                 |
| None                     | 72 x 10^4             | 58 x 10^4       |
| None                     | 60 x 10^4             | 48 x 10^4       |
| Interferon (1000 U/ml)   | 80 x 10^4             | 33 x 10^4       |
| Interferon               | 49 x 10^4             | 40 x 10^4       |

carbon hydroxylase in hamster cells (D. Nebert, personal communication) are also blocked by MPB. All of these actions may be membrane-associated phenomena.

The current concept of how interferon brings about an antiviral state in cells is summarized in Fig. 4. Interferon is bound to the cell membrane. This action may be reversed by trypsin treatment. A step follows which is the site of inhibitory action by MPB. This may involve entry of interferon into the cytoplasm. Cell RNA and protein synthesis then appear to be required. These are blocked by, among other inhibitors, actinomycin D and puromycin or fluorophenylalanine, respectively. One possibility is that the actual antiviral activity may be the result of inhibition of viral protein synthesis caused by a specific cell protein. Evidence that viral protein synthesis is indeed the site of interferon action is presented in the following section.

Site of Antiviral Action

The observations just described provide no information about which stage in the virus growth cycle is inhibited in the interferon-treated cell. To investi-
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gate this, detailed biochemical studies have been performed on the effects of interferon on events taking place within the cell during the replication of RNA and DNA viruses.

**DNA Viruses**

Most of the work investigating the antiviral action of interferon in DNA virus replication has employed vaccinia virus in chick or mouse cells as a test system. Vaccinia is a large virus with a complex internal structure containing DNA, lipids, and structural proteins. Some of the structural proteins of the virion are functional including a viral DNA-dependent RNA polymerase (29, 30).

Preliminary uncoating of vaccinia virus takes place in the cytoplasm where all events in the viral replication cycle appear to occur (31). This step takes place normally in interferon-treated cells (32). The complete uncoating of the viral nucleocapsid is a complex process which is partially blocked in the interferon-treated cell (32). This step involves the elaboration of a messenger RNA by the polymerase of the virus particle. This messenger RNA codes for the formation of an uncoating enzyme which releases the genome of the virion. Thus, the parental genome is freed for subsequent steps in the replication process. Virus-directed protein synthesis is therefore important for the final uncoating of the genome (33); however, transcription of the parental genome can take place before complete uncoating of the virus. In fact, it has been shown that in the absence of protein synthesis, transcription of the parental genome takes place in an uncontrolled manner leading to increased production of viral messenger RNA (34).

In an extensive study of the effect of interferon on vaccinia virus replication...
in mouse L cells, Joklik and Merigan (35) found an inhibition of DNA polymerase and of viral DNA production in interferon-treated cells. Paradoxically, they reported that the production of viral messenger RNA was increased, and, although the half-life of the viral messenger RNA was unaltered, it did not combine with ribosomes to form polyribosomes. In fact, a disaggregation of host cell polyribosomes had taken place, and interferon-treated cells were almost devoid of both host and viral polyribosomes. Marked cell destruction was noted in the interferon-treated cells. They (35) therefore concluded that a large amount of vaccinia virus messenger RNA was produced but was not translated in interferon-treated cells and that the mechanism of action of interferon was on a step between transcription and translation of virus genetic information.

Subsequent studies have confirmed the conclusions of Joklik and Merigan (35) and served to explain some of their puzzling findings. An RNA polymerase is now known to be a structural element of the virion (29, 30), and its production of viral RNA is increased in the absence of viral protein synthesis (30). In the interferon-treated cell, therefore, the uncontrolled transcription of parental DNA can be explained. The inhibition of cellular protein synthesis can be accounted for as this is due to another structural element of the virion (36). Since the uncoating protein appears to be a virus product, its synthesis would be expected to be blocked in interferon-treated cells and as a consequence of this, final virus uncoating inhibited. Most of the viral DNA does remain within the nucleocapsid in interferon-treated cells (32).

The mechanism of action of interferon on the early steps in vaccinia virus replication is shown in Fig. 5. The virus enters the cell (Fig. 5, 2) and loses its outer coat (Fig. 5, 3). The nucleocapsid which remains (Fig. 5, 4) produces vaccinia virus messenger RNA since a viral polymerase is present within the core structure (Fig. 5, 5). Vaccinia messenger RNA then forms polyribosomes with cellular ribosomes (Fig. 5, 6). A step between Fig. 5, 5 and Fig. 5, 6 is blocked in interferon-treated cells since viral polyribosomes do not form. In normal infection a viral protein is produced (Fig. 5, 7) which uncoats the viral nucleocapsid (Fig. 5, 8) to release viral DNA (Fig. 5, 9). In the interferon-treated cell, vaccinia messenger RNA accumulates, and the DNA remains within nucleocapsids.

The effect of interferon on the activity of another DNA virus, SV40, has been extensively studied. The transformation of the 3T3 line of mouse cells (37) and the production of SV40 neoantigen, T antigen, are inhibited by interferon (38). In the case of cells already transformed by SV40, however, T antigen production is unaffected by interferon treatment. The cells, however, remained sensitive to interferon (5).

Important studies were also performed on an adenovirus-SV40 hybrid.
Adenoviruses are much less sensitive to interferon than are SV40 replication and T antigen production (39), and in interferon-treated cells coinfectected with both SV40 and adenovirus 7, T antigen production by SV40 was inhibited (40). In cells infected with the adenovirus-SV40 hybrid virus, however, T antigen production was insensitive to interferon pretreatment (40). Therefore, in two situations the sensitivity to interferon pretreatment of SV40-T antigen production was altered. In one case this was found in cells with a portion of the SV40 DNA probably integrated into the host genome (39); in the other, it was found in cells with a portion of the SV40 DNA integrated into the genome of an interferon-insensitive virus (40).

**RNA Viruses**

The best understood systems employing RNA viruses for the study of interferon action are the growth of the Group A arbovirus Semliki Forest virus (SFV) in chick cells and of mengovirus in mouse cells. All studies agree that the primary site of interferon action is on an early event in the virus replication cycle.

After adsorption and penetration the single-stranded RNA of both viruses is released (Fig. 6, 3). The input viral RNA must immediately act as a genetic messenger (Fig. 6, 4) since no RNA-dependent RNA polymerase exists in the host cells or in the virions. The production of the viral RNA polymerase is the key event in the early steps of virus replication (Fig. 6, 5). Once this enzyme is produced a complementary or negative strand of viral RNA can be formed (Fig. 6, 6). The viral polymerase uses the negative strand of viral RNA as a template to turn out strands of progeny viral RNA (Fig. 6, 8).
During this process a partially double-stranded RNA complex is formed due to the displacement from the duplex of completed viral RNA forms by newly initiated RNA strands (Fig. 6, 7).

Progeny RNA can serve one of at least three functions: it may be incorporated into virions, or it may serve as a messenger RNA form coding for structural and functional viral proteins, or it may act as a template for the production of additional negative strands. Initiation of viral RNA replication is therefore dependent both on translation of input RNA to form an RNA polymerase, and on the activity of this enzyme in transcribing the RNA.

Which of the early steps in virus replication is blocked by interferon? Virus adsorption is unaffected (41-43). The dissociation of viral RNA from its protein coat is not impaired in the interferon-treated cell since virus growth following infection with uncoated viral RNA is also inhibited (44). This means that the sensitive step in the growth cycle of RNA viruses is beyond the adsorption, penetration, and uncoating stages (after step 3, Fig. 6). Inhibition of progeny viral RNA synthesis in interferon-treated cells has been demonstrated in several systems (45-47). The formation of all classes of SFV RNA in chick cells was also found to be depressed by treatment with high titers of interferon. This included the production of a ribonuclease-resistant viral replicative form (48). Gordon et al. (49), made similar observations in the mengovirus-mouse cell system.

In addition to inhibition of production of progeny viral RNA, interferon treatment blocks the synthesis of virus-specific proteins. Production of the polymerases of both SFV (50) and mengovirus (51) was inhibited in interferon-treated cells. As both studies were performed late in infection, most of the enzyme produced was probably made by translation of progeny viral RNA. Since the production of progeny viral RNA was inhibited in interferon-
treated cells, it is likely that the observed inhibition of the polymerases was a consequence of the effect on viral RNA synthesis rather than being a primary cause of inhibition of virus replication.

Studies have therefore focused on the function of the parental genome. In chick cells treated with very high titers of interferon radioactive input SFV RNA was not converted into a ribonuclease resistant replicative form (Fig. 7 A and D). This suggested that interferon-treated cells did not produce strands of RNA complementary to the parental genome. Therefore, transcription of the parental RNA did not take place. In this respect previous incubation with interferon was similar to treatment with general inhibitors of protein synthesis such as puromycin and cycloheximide (Fig. 7 B and C) which also block the transcription of parental viral RNA, presumably by inhibiting the translation of parental RNA, i.e., the synthesis of the viral RNA polymerase.

In one respect, however, interferon action does differ from that of general
inhibitors of protein synthesis. In SFV-infected chick cells, parental viral RNA enters into a membrane-bound replicative complex (RC) which contains the replicating forms of the viral RNA (Fig. 8 A). Later in infection this RC has been shown to contain the viral RNA polymerase (52). Cyclo-

heximide treatment inhibits entry of viral RNA into this complex (Fig. 8 C). On the other hand, treatment with interferon has no effect on the association of parental viral RNA with the RC (Fig. 8 B); however, only single-stranded viral RNA is found in association with the RC under these conditions (R. M. Friedman and T. Sreevalsan, data in preparation) as the replicating forms cannot be produced in interferon-treated cells (Fig. 7 C).
It was of interest to determine directly whether interferon treatment had any inhibitory effect on the translation of the input RNA virus genome. At least five virus-specific proteins could be detected by polyacrylamide gel electrophoresis in the cytoplasm of SFV-infected chick cells (Fig. 9). Two of these proteins were clearly structural proteins of the virion (53), and synthesis of these proteins by parental viral RNA was observed in infected cells incubated at 37° (Fig. 9) or 42°C (Fig. 10). At 42°C viral RNA synthesis was
inhibited by 87% (Table V). This treatment, however, had little effect on the amount of virus protein made early in infection. That similar results with respect to virus protein synthesis were obtained at both 37°C and 42°C therefore indicates that the bulk of early viral protein synthesis is directed by the parental genome. In interferon-treated cells no virus-specific proteins could
be detected at 37° (Fig. 9) or at 42°C (Fig. 10). This result indicates that parental viral RNA is not translated in interferon-treated cells (54).

The in vivo studies of Levy and Carter (55) have suggested that the anti-
viral state results from an alteration of 40S ribosomal subunits in interferon-
treated cells so that they cannot interact with input viral RNA. At 30 min
after infection some radioactive input viral RNA sedimented at 50S in sucrose
gradients. This peak was absent in gradients made from extracts of cells
reated with interferon before infection. Later in infection in controls, a
decrease was noted in the number of counts present in the 50S component
and an increase in those associated with a 240S component; the latter is
thought to represent viral polyribosomes. In interferon-treated cells some
240S component was present, but still no 50S component was seen. The
authors concluded that the 50S complex was due to an interaction of viral

| Specific activity | Incubation temp. | RNA   | Protein |
|-------------------|------------------|-------|---------|
|                   | °C               | cpm/μg protein |         |
| 37                | 15.9*            | 12.1  |         |
| 42                | 2.0              | 18.8  |         |

* Actinomycin D-treated (2 μg/ml) cells were infected with virus in the
  presence of guanidine (3 mg/ml). After washing, the cells were incubated for
  30 min at 37° or 42°C in Eagle's medium. The cells were then pulse labeled for
  10 min with 10 μCi of uridine-3H or leucine-3H per ml. Total protein and
  acid-precipitable radioactivity were then determined.

RNA with a 40S ribosomal subunit, that this was necessary to form a func-
tional polyribosome, and that interferon treatment prevented this interaction.
The nature of the 50S complex was not, however, clearly established, and to
this reviewer it remains an open question whether the ribosomal subunit is
the primary site of the interferon action.

The results of studies of interferon action on RNA virus replication are
summarized in Fig. 6. They suggest a block between steps 3 and 4. While
this seems to specify a distinct site of action, it really does not since this is the
step which involves initiation of animal virus protein synthesis about which
very little is known.

Results of studies in cell free systems will be discussed by Dr. Sonnabend in
the following report.

**DISCUSSION**

Studies on the development of resistance to virus infection following interferon
treatment clearly establish at least three distinct cellular activities which are
required for this activity. Interferon binding to the cell must take place. Work with MPB implies a requirement for a step after binding, perhaps involving entry of interferon or modification of the cell. The third step is cellular macromolecule synthesis. Data on the requirement for RNA synthesis seem quite convincing, but, as discussed below, the requirement for protein synthesis is not so well established.

The particular species of RNA required for interferon action is not known. Results from my laboratory with various doses of actinomycin D indicate that a concentration of 0.3 μg/ml which completely inhibits interferon action in chick cells still permits some ribosomal RNA synthesis. Taken together with a report that puromycin aminonucleoside (which selectively inhibits ribosomal RNA synthesis [56]) had no effect on interferon action (23), it would seem that ribosomal RNA is not the species required. No data which would establish a requirement for transfer RNA, messenger RNA, or any other cell RNA species are available. It is also not certain that the species of RNA required is unique in the sense that it is induced by interferon treatment.

Since the species of RNA which is required for interferon action is unknown, it is difficult to deliberate on this RNA's function. Reasonable speculation about this problem is closely related to the question of whether interferon is taken up by cells. If interferon acts from an external location (presumably its site of attachment on the plasma membrane) certain possible mechanisms of interferon action would be unlikely. For instance, the RNA form required for interferon action could be employed to transport interferon into the cell or to modify it once there; however, the likelihood of these possibilities is, of course, nil if interferon acts from an extracytoplasmic position.

The data on the requirement for cell protein synthesis for interferon action are not as convincing as those establishing the need for RNA synthesis by use of actinomycin D. This is because one must reverse the inhibition in order to permit virus growth. Also the dose of inhibitor used must not block cellular RNA synthesis, a condition which has been met in only a few reports. However, even if these conditions are fulfilled, a direct need for a protein per se is not necessarily established. For instance, cycloheximide has little effect on ribosomal RNA synthesis but does inhibit the formation of ribosomes, presumably by blocking the synthesis of a protein required for their maturation (57). Thus, one effect of an inhibitor of a protein synthesis might be to block the formation of a ribonucleoprotein or of an RNA species.

If a protein is, however, required for interferon action, a strong possibility is that the species of RNA also required is the messenger for that particular protein. A model for the development of the resistant state based on this notion was suggested by Taylor (18). She proposed that interferon acts as a derepressor; therefore, treatment of a cell with interferon would be followed by synthesis of a specific messenger RNA directing the synthesis of a protein possessing antiviral activity.
As will be discussed in detail by Dr. Sonnabend, Marcus and Salb (58) suggested that the site of action of this postulated antiviral protein was the ribosome. The cellular antiviral protein was thought by them to interact with the ribosome to prevent the synthesis of viral proteins either by preventing the attachment of the viral messenger RNA to ribosomes or the translation of the bound messenger RNA. This work has many interesting implications, far beyond its possible importance in uncovering the mechanism of action of interferon. It is, however, based on the unproved theory of Taylor that interferon induces a specific new antiviral protein. Furthermore, experiments by Kerr et al. (59) and R. Z. Lockart (personal communication) have so far failed to confirm the findings of Marcus and Salb (57). In addition, E. M. Martin, I. M. Kerr, and J. A. Sonnabend (personal communication) and G. Bodo (personal communication) have been unable to obtain evidence for the association of a newly synthesized protein with ribosomes in cells treated with interferon. These findings taken together must bring into question the proposals of Marcus and Salb (57).

With respect to the expression of resistance in the interferon-treated cell, the experiments discussed point to an early event in the virus replication cycle as the site of action of interferon. It is evident that interferon has no effect on steps up to and including virus uncoating except in the case of vaccinia where complete uncoating is related to virus protein synthesis. It also seems clear that transcription of viral DNA occurs in interferon-treated cells, but translation of viral RNA (input DNA in the case of single-stranded RNA viruses, messenger RNA in the case of DNA viruses) does not occur. Inhibition of other events in the virus replication cycle would appear to follow as a consequence of an inhibition of the translation of viral messenger RNA.

The basic problem in understanding interferon action is therefore how interferon blocks viral protein synthesis and yet does not seem to effect total host protein synthesis significantly. The data of Joklik and Merigan (34) indicate that virus messenger RNA does not combine with ribosomes to form polyribosomes in interferon-treated cells. This in turn means an inhibition of the initiation of virus protein synthesis. Since this must be a very complex reaction, I would like to discuss briefly the factors which are obviously involved in order to arrive at what are reasonable guesses as to the site of interferon action. These factors include viral messenger RNA and cellular ribosomes, energy generating systems, transfer RNA forms, and protein synthesis initiation factors.

Of these, viral messenger RNA must be involved. In fact it is possible that interferon or some factor induced by it is able to interact or to modify viral RNA directly and thus render it incapable of combining with ribosomes. No modification of cellular constituents would then be required. Findings on infection with the adenovirus-SV40 hybrid and the SV40 transformed cell are of considerable interest with respect to this hypothesis (40). Since the
synthesis of an SV40 protein normally inhibited in interferon-treated cells is not blocked in the above situation, there can be nothing in the primary structure of the viral messenger RNA that prevents its translation in interferon-treated cells. Resistance or sensitivity to interferon must therefore be determined by such characteristics of viral messenger RNA as conformation or the nature of the specific regions employed for attachment to ribosomes and initiation of protein synthesis.

It is of course also possible that interferon causes alteration in one of the other factors mentioned, which in turn renders it incapable of reacting productively with viral RNA. Whatever the modification induced, the factor must still be capable of being employed for cell protein synthesis. Thus, a basic difference between viral and cellular messenger RNA can be exploited to the detriment of the virus.

The energy generating systems of the cell would seem an unlikely site for interferon action as it is difficult to conceive of a method to make them available to the cell and not to the virus. Another possible site is on transfer RNA. If the initiating codons of the virus and the cell differ, changes induced by interferon in transfer RNA forms involved in initiation might seriously inhibit virus protein synthesis. It is also possible that the site of interferon action could involve a chain initiating factor for protein synthesis. Very little is known yet about such factors in animal cells, but they might be able to distinguish between viral and cellular RNA.

The ribosome has been indicated by two laboratories to be the primary site of interferon action (54, 57). It is possible in fact that an initiating factor acting in conjunction with a ribosome is responsible. Although the experiments which suggested that the ribosome is the primary site of interferon action are being actively discussed at present, the hypothesis certainly still remains an attractive one.

At any rate, we are still far from understanding interferon action completely. As a logical start in this direction a cell-free system producing bona fide viral polypeptides will be necessary in order to dissect the factors required for initiation of virus protein synthesis and to pick the one directly involved in interferon action.

From what has been uncovered so far about interferon action some reasonable notions may be put forward to account for its properties. The inhibition of a wide range of RNA and DNA viruses is undoubtedly due to its effect on translation of viral messenger RNA. The selectivity of interferon, that is the ability to distinguish between viral and host messenger RNA's, is probably due to basic differences between these. It is also possible, though not fashionable, to account for this by postulating that virus protein synthesis proceeds by a mechanism basically different from that of the host cell. Finally, it is possible but also unlikely that interferon action is selective only in the sense that it
inhibits the initiation of all new protein synthesis, viral and cellular. The variation in virus sensitivity might therefore be due to the fact that some viral messenger RNA's resemble those of the cell more so than others, or that protein synthesis in insensitive viruses is similar to that of the cell.

The species specificity of interferon is most likely caused by factors relating to the binding and possible entry of interferon or to the ability of interferon to act as an inducer of a specific cell protein or modifier of an already existing cell element. Finally, the remarkable potency of interferon could also be due to an inducer or enzymatic function of interferon once processed in the proper cell.

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