Development of the Antiviral State in Response to Interferon

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ABSTRACT The development of resistance in response to interferon depends on cellular RNA synthesis and probably also on cellular protein synthesis. The evidence for these requirements is reviewed, as well as the proposal that this evidence indicates the existence of a *specific* response of the cell to interferon, involving the induced synthesis of an antiviral protein. Direct evidence for such an interpretation has not been obtained, and alternative explanations are discussed which do not require quantitative or qualitative differences in the RNA and protein made in cells exposed to interferon. The possible role of the ribosome in the antiviral action of interferon is also discussed.

When Dr. Merigan asked for a discussion of an aspect of interferon action that was controversial, the selection of such a topic seemed to present little difficulty. However, the topics I will talk about are two which appear to be in danger of becoming noncontroversial. The first relates to the development of resistance and the significance of the requirement this has for cellular RNA and protein synthesis. The second topic I will talk about concerns the possible role of the ribosome in the antiviral action of interferon.

With respect to the development of resistance, the question I would like to consider is whether interferon action involves a specific derepression. In other words, do cells exposed to interferon make a specific messenger RNA directing the synthesis of a protein which is the effective antiviral agent? Dr. Friedman has more fully discussed the evidence for such a mechanism, which was first proposed by Taylor (1964) on the basis of her finding that actinomycin D blocked the development of resistance in response to interferon. The reduction in single-cycle virus yields in interferon pretreated cells was not observed when this pretreatment took place in the presence of actinomycin. Actinomycin added after interferon did not have this effect. It was therefore the development of resistance, but not its expression, that was sensitive to actinomycin. These observations established that interferon action has a requirement for cellular RNA synthesis. They, however, provided no information

about which species of RNA was involved. Puromycin aminonucleoside which selectively inhibits the formation of ribosomal RNA (Farnham and Dubin, 1965) had no effect on interferon action (Field et al., 1967), suggesting that it is the synthesis of messenger RNA or transfer RNA that may be required for the development of resistance. Having demonstrated that the development of resistance depends on cellular RNA synthesis, the next step was to try to show that there was a similar dependence on cellular protein synthesis. Experiments on the effects of inhibitors of protein synthesis present some problems about which I will speak a little later, and which have made the interpretation of the results of these experiments more difficult. Nonetheless, although less clear cut than the observations on the effects of actinomycin, studies with several inhibitors of protein synthesis have suggested that the development of resistance in response to interferon may also have a requirement for host cell protein synthesis (Friedman and Sonnabend, 1964, 1965; Lockart, 1964; Levine, 1964; Dianzani et al., 1969).

It has been assumed that the fact that host cell macromolecular synthesis is required for the development of resistance means that there is a *specific* cellular response to interferon. In other words, cells exposed to interferon synthesize a specific protein; it has been further assumed that this protein is the effective antiviral agent.

The evidence needed to show that these hypotheses are correct is, first, a direct demonstration that interferon-treated cells contain a newly synthesized protein that is absent—or present in relatively smaller amounts—in untreated cells, and that this protein has antiviral activity. Despite the obvious difficulties, our group at Mill Hill undertook an examination of interferon-treated chick cells for the presence of a protein or proteins which are either absent or present in untreated cells in reduced amounts.

Our approach was to incubate interferon-treated and untreated cells with the same amino acids but labeled with different isotopes, usually ^{3}H in the case of the untreated cells, and ^{14}C in the case of interferon-treated cells. The ^{3}H- and ^{14}C-labeled cells were mixed, and various subcellular fractions were prepared. The proteins in the various fractions were treated with 1% SDS and 0.5 M urea and electrophoresed together on 10% polyacrylamide gels. An alternative technique was to incubate the cells with amino acids-^{3}H before exposure to interferon then with amino acids-^{14}C during incubation with interferon or with buffer. The experiments were carried out on 2–3-day-old monolayers of chick embryo cells using levels of interferon from 30 to 100 U/10^{7} cells, with periods of incubation in the presence of interferon of from 5 to 21 hr. The partially purified interferon was kindly supplied by Dr. Karl Fantes and had a specific activity of 10^{4}–10^{5} U/mg protein. Particular attention was paid to the ribosomes, in view of the reports of Marcus and Salb (1966) and of Carter and Levy (1967) implicating these structures in the

antiviral action of interferon. Both native ribosomal subunits and subunits derived by treatment of 74S ribosomes with EDTA were examined, as well as the proteins that could be removed from the ribosomes with 0.2 M NaCl and 0.01 M EDTA. In preliminary studies, it had been found that the sedimentation in sucrose gradients of ribosomes and their subunits from interferon-treated cells did not differ from that of ribosomes from untreated cells; nor were there differences in the yield of ribosomes, the ratio of monosomes to polysomes, and the density of the ribosomes in caesium chloride. Cell sap proteins were subjected to various preliminary fractionation procedures before electrophoretic analysis, and these included precipitation with different concentrations of ammonium sulphate and fractionation on DEAE-cellulose columns.

Despite many experiments under a variety of conditions we were unable to detect a consistent difference in the material derived from interferon-treated cells. An example of the patterns obtained on electrophoresis of proteins derived from the 60S ribosome subunit is shown in Fig. 1.

Material derived from interferon-treated cells was labeled with amino acids ^{14}C, that from control cells with amino acids-^{3}H. The ratio of ^{14}C to ^{3}H radioactivity is identical. All the other fractions examined gave no consistent evidence of a specific newly synthesized protein in material from interferon-treated cells.

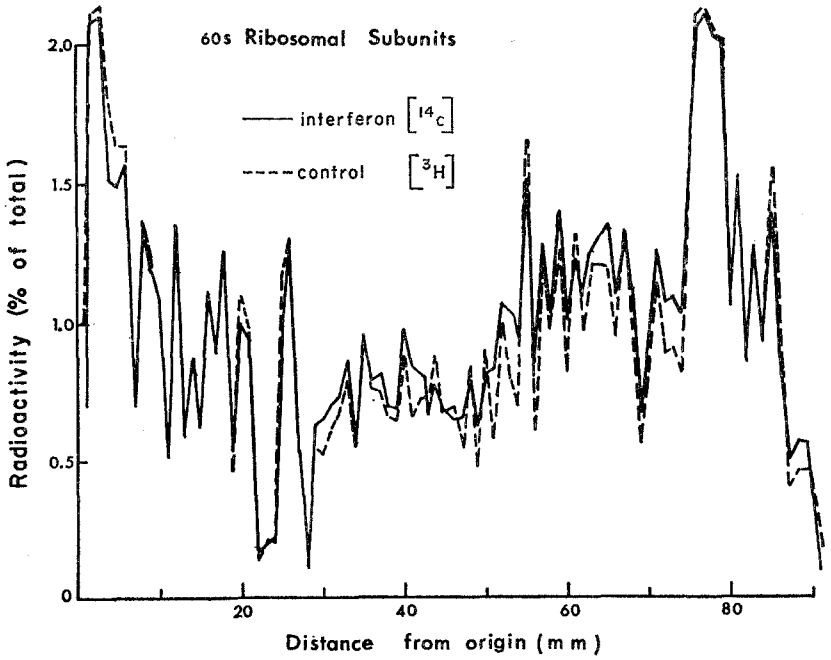

FIGURE 1. See text for explanation.

Our negative results raise the question of whether we should in fact be able to detect the presence of one additional protein probably attached to only a proportion of the ribosomes. We have calculated that if one-third of the ribosomes in the resistant cell contain a single newly synthesized antiviral protein molecule, and if the size and degree of labeling of this protein is the same as that of one average newly synthesized ribosomal protein, then we should have been able to detect its presence, even if it had the same electrophoretic mobility as the major group of ribosomal proteins. However, small differences in these assumptions would affect this conclusion so our negative results cannot, therefore, rule out the possibility that a newly synthesized protein is associated with ribosomes in interferon-treated cells.

I would now like to return to a discussion of some of the considerations on which the proposal that interferon induces the synthesis of a protein possessing antiviral activity is based and ask two questions. First, how good is the evidence that protein synthesis is required for interferon action, and second, if this should be a reasonable interpretation, what evidence do we have that it is the induced synthesis of a specific protein that is required rather than the continuation of normal cellular protein synthesis?

With regard to the first question, there are some problems inherent in experiments with inhibitors of protein synthesis that are difficult to overcome. Unlike actinomycin, which does not inhibit the replication of most RNA viruses, it is necessary to reverse the effects of the inhibitor of protein synthesis when the virus is added, in order to determine whether the interferon treatment has been effective. Furthermore, it is necessary to show that the inhibitor does not suppress RNA synthesis, otherwise we shall have advanced no further than the observations on the effects of actinomycin. Concentrations of puromycin, for example, have been used in some of these experiments which do inhibit RNA synthesis, and reversal of the inhibitor with respect to the effects of pretreatment on subsequent virus growth has not always been complete. This means that the effectiveness of interferon is being compared with a control which is itself, at least partially, inhibited. Unless these criteria, reversibility with respect to virus growth and no inhibition of RNA synthesis, are met, interpretation of the inhibitor studies will be difficult. These criteria have been met by using low doses of puromycin, doses which inhibited protein synthesis by only 50% (Friedman and Sonnabend, 1965), with fluorophenylalanine (FPA) (Friedman and Sonnabend, 1964; Baron et al., 1967), and with cycloheximide (Dianzani et al., 1969). However in one detailed study, cycloheximide failed to inhibit the development of resistance (Dianzani et al., 1969). It was suggested that the reason for this was that the antiviral protein was rapidly made when the inhibitor was removed, the messenger RNA for this protein having been synthesized while the cells were exposed to interferon in the presence of cycloheximide. The difficulty with this explana-

tion is that it implies that it is impossible to ever demonstrate the need for new protein synthesis for the development of resistance, using an inhibitor that readily fulfills the criteria mentioned; that is, it is easily reversible—protein synthesis is rapidly restored on its removal, and it does not inhibit RNA synthesis. The inhibitor will not affect the development of resistance precisely because it fulfills these necessary criteria. In the case of puromycin and FPA, inhibitors that apparently prevent the development of resistance, it has required special pleading about the instability of the messenger RNA in the presence of these inhibitors to account for their effects as opposed to the lack of effect of cycloheximide.

Because of the above considerations, there is a need for some caution in the interpretation of the results of these experiments, although the evidence they have provided strongly suggests that the development of resistance has a requirement for cellular protein synthesis.

Accepting the validity of the inhibitor studies, in that they show that interferon action has a requirement for cellular RNA and protein synthesis, I would like to consider the second question I asked. Does the requirement for RNA and protein synthesis reflect the induced synthesis of a *specific* protein in response to interferon? The demonstration that interferon action depends on RNA and protein synthesis does not in itself permit such a conclusion. An alternative explanation would be that interferon action requires the continuation of normal cellular RNA and protein synthesis. There need be no quantitative or qualitative differences in the RNA and protein made while cells are exposed to interferon. For example, interferon itself may be antiviral within the cell, but its uptake or transport to its site of action may depend on cellular RNA and protein synthesis. Alternatively, these processes may be needed for the activation of interferon, or for its modification. One argument for the derepressor model of interferon action is its high potency and that such a model provides an explanation of how its effects could be amplified. However, an amplification effect could also result from mechanisms which do not require that interferon action depend on the induced synthesis of a specific protein. For example, a mechanism involving interferon as an enzyme, or as an agent triggering the release or activation or modification of a preexisting cellular enzyme, could have this result in that it is the product of the reaction catalyzed by this enzyme that is responsible for the antiviral state. A specific example would be a modification of a newly made tRNA. In this case the need for new RNA synthesis could be accounted for by the fact that it is only newly synthesized tRNA that is susceptible to modification.

Thus it is possible to interpret experiments employing inhibitors to indicate that the protein and RNA synthesis involved in interferon action are neither specifically induced by interferon nor necessarily antiviral.

I would now like to turn to a different aspect of interferon action, and this concerns the possible role of the ribosome in the expression of the antiviral state.

Marcus and Salb in 1966 and Carter and Levy in 1967 provided evidence that the antiviral action of interferon was the consequence of a specific ribosomal abnormality induced by treatment of cells with interferon. Ribosomes from interferon-treated cells were unable to translate a viral messenger RNA while retaining the ability to translate cellular messenger RNAs. The work of Marcus and Salb indicated that there were in fact two ribosomal defects: a reduced capacity to bind viral RNA and an inability to translate the RNA that was bound. In the system studied by Carter and Levy, the defect was in the binding of viral RNA, and the inability to translate was regarded as a consequence of this. Linking their proposals with the derepressor model for the development of resistance, Marcus and Salb assigned a specific role to the new protein that was believed to be made in response to interferon. This protein was termed the "Translational Inhibitory Protein" or TIP, and Marcus and Salb suggested that the TIP associated with ribosomes to cause the functional defects they described. They provided some evidence that a protein was involved in the abnormal ribosomal function in their demonstration that treatment of the ribosomes with trypsin restored their apparent capacity to translate viral RNA without improving their ability to bind the RNA. That the TIP was a protein newly synthesized on exposure to interferon was assumed because the ribosomal abnormality was not observed when cellular RNA synthesis was blocked by actinomycin D during treatment with interferon.

These results prompted us to a detailed examination, at Mill Hill, of the interaction of viral RNA and ribosomes in a cell-free amino acid incorporating system derived from CEF (chick embryo fibroblasts) and employing techniques similar to those used by Marcus and Salb.

First, ribosome and cell sap preparations from interferon-treated and untreated cells were compared with respect to their ability to support amino acid incorporation, either endogenous incorporation by a total ribosome preparation, or poly U-stimulated incorporation of phenylalanine by fractionated 74S ribosomes.

A comparison of incorporation by systems derived from interferon-treated and untreated cells is shown in Table I.

Partially purified interferon of a specific activity of 10^4–10^5 U/mg protein, which was kindly supplied by Dr. Karl Fantes, was used at concentrations of 50 or 140 U/ml. 2-day-old CEF monolayers were used, and the interferon treatment was for 24 hr. The intrinsic incorporation by a total ribosome preparation is seen in this table, and no differences were observed in the

activity of ribosome or cell sap preparations derived from interferon-treated or untreated cells.

Table II shows the response of fractionated 74S ribosomes to poly U, and again ribosomes from interferon-treated and untreated cells were equally active.

The interaction of these ribosomes with viral RNA was next examined. ^{3}H-labeled Sindbis viral RNA or Semliki Forest virus RNA as well as 45S

TABLE I

AMINO ACID INCORPORATION BY RIBOSOME–CELL SAP SYSTEMS FROM INTERFERON-TREATED AND CONTROL CELLS

Ribosomes	Cell sap	Incorporation of amino acid-^{14}C mixture			
		Exp. 1	Exp. 2	Exp. 3	Exp. 4
		cpm/50 μg ribosomes			
Control	Control	5740	6100	3500	6200
Interferon*	Control	6200	5900	4750	6400
Interferon‡	Control	—	—	—	6200
Control	Interferon*	—	5950	—	6650
Control	Interferon‡	—	—	—	7300
Interferon*	Interferon*	—	6300	—	6200
Interferon‡	Interferon‡	—	—	—	6300

* Interferon treatments of cells were at concentrations of from 35 to 50 U/ml.
‡ Interferon treatments of cells were at a concentration of 140 U/ml.

TABLE II

RESPONSE TO POLY U OF CELL-FREE SYSTEMS FROM INTERFERON-TREATED AND CONTROL CELLS

Ribosomes	Cell sap	Poly U	Phenylalanine-^{14}C incorporation	
			No poly U	+ Poly U
		μg/0.5 mg ribosomes	*cpm/0.5 mg ribosomes*	
Control	Control	12.5	1100	10,650
		20	—	9900
Interferon	Control	12.5	1000	9900
		20	—	9360

and 26S SFV RNA derived from infected cells were used in different experiments. The pattern of the experiments followed closely that described by Marcus and Salb (1966). Radioactive viral RNA was mixed with fractionated single ribosomes in the cold, and after holding the mixture at 0°C for 45 min in a complete amino acid incorporating system, the fraction of viral RNA bound to ribosomes was analysed by sedimentation of the mixture in a sucrose gradient.

The results with labeled viral RNA from different sources is shown in Table

III. The fraction of the RNA bound is that proportion of the added labeled RNA sedimenting at or more rapidly than 74S. Although there are some variations in the absolute amounts of RNA bound in different experiments, no differences were noted in the relative amounts bound to ribosomes from interferon-treated or untreated cells.

The appearance in sucrose gradients of complexes formed by the viral RNA and ribosomes is shown in Fig. 2.

Sedimentation is from left to right. The optical density trace is that given by the ribosomes. EMC virus was included as a sedimentation marker. The labeled RNA used in this experiment was a mixture of 45S and 26S SFV RNA extracted from infected cells. The RNA does form complexes, and a substan-

TABLE III

BINDING OF ^{3}H-LABELED VIRAL RNA TO RIBOSOMES IN THE CELL-FREE SYSTEM

RNA preparation	Ribosomes	RNA bound to ribosomes	
			Fraction of added RNA
		cpm	%
SFV RNA (26 + 45S)	Control	14,300	58
	Interferon	19,070	81
SFV RNA (45S)	Control	750	48
	Interferon	562	36
Sindbis virus RNA	Control	1580	76
	Interferon	1540	72
Sindbis virus RNA	Control	555	61
	Interferon	694	77
Sindbis virus RNA	Control	2361	77
	Interferon	2395	78

tial fraction of the RNA runs as a broad shoulder on the heavy side of the 74S ribosome peak. On no occasion have we been able to observe a discrete 250S structure such as that described by Marcus and Salb and which was regarded as a specific viral polysome that had been formed in vitro in the cold. When ribosomes from interferon-treated cells were used the pattern seen on sucrose gradient analysis was identical, and as already mentioned, the same amount of viral RNA associated with ribosomes derived from interferon-treated cells. RNA extracted from purified Sindbis virus or SFV RNA isolated from infected cells gave similar results in experiments of this type.

The evidence presented by Marcus and Salb, that ribosomes from interferon-treated cells were unable to translate viral messenger RNA's, was based on the interpretation that breakdown at 37°C of the 250S structure formed in the cold between ribosomes and viral RNA was the result of translation of the viral RNA. The fact that the complexes formed with ribosomes from inter-

feron-treated cells did not break down on incubation indicated that such ribosomes were unable to translate the viral messenger RNA. Although we were unable to demonstrate a discrete 250S structure in sucrose gradient analyses of the complexes formed between ribosomes and viral RNA in the cold, we did look at the fate of the complexes that were formed when these were incubated at 37°C.

Fig. 3 shows the result of such an experiment.

The ribosomes were derived from interferon-treated cells in this experiment, and the RNA used was ^{3}H-labeled SFV RNA. Identical results were obtained when ribosomes from untreated cells were used. The upper curve shows the complexes formed after a 25 min incubation of the RNA and ribosomes at 0°C. The lower curve shows that the complexes break down on incubation at 37°C. This incubation at 37° was also carried out in the presence of cyclo-

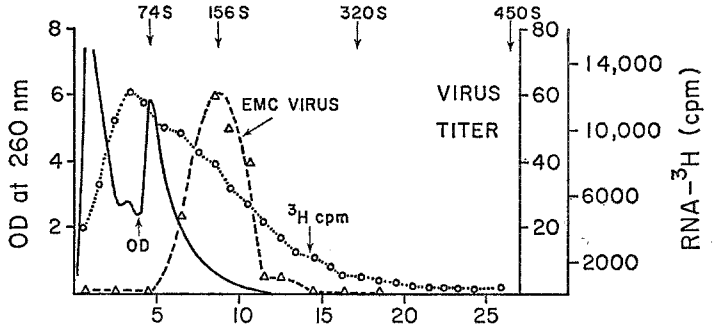

FIGURE 2. Sucrose gradient analysis of complexes formed on incubation at 0°C in the cell-free system of SFV RNA and 74S chick cell ribosomes. —, optical density at 260 nm; △--△, EMC virus titer, (HAU); ○···○, ^{3}H-labeled SFV RNA.

heximide, and in a system where ATP, GTP, and phosphoenolpyruvate were omitted. It can be seen that the complexes broke down to the same extent under these conditions. That these treatments were effective in inhibiting amino acid incorporation is shown in the lower diagram; in this case, endogenous incorporation by a total ribosome preparation was assayed under the same conditions as the incubations shown in the upper diagram.

Since breakdown took place under conditions where protein synthesis was inhibited, it could not be taken as a measure of translation. Ribonuclease activity could be demonstrated in ribosome and cell sap fractions, and it is likely that this contributed to the breakdown of the complexes we observed.

These results are in conflict with those of Marcus and Salb (1966) and of Carter and Levy (1967) in that they show no differences in the capacity of ribosomes from interferon-treated and untreated cells to bind viral RNA. With respect to translation of the viral RNA, we have been unable to use the index of translation of Marcus and Salb, namely the breakdown at 37°C of

RNA ribosome complexes formed in the cold, as we have found that this could not be correlated with amino acid incorporation.

In vivo studies on the growth of both RNA and DNA viruses have all pointed to viral protein synthesis as the function sensitive to inhibition in interferon-treated cells, and indeed, as pointed out by Joklik, (1967) this

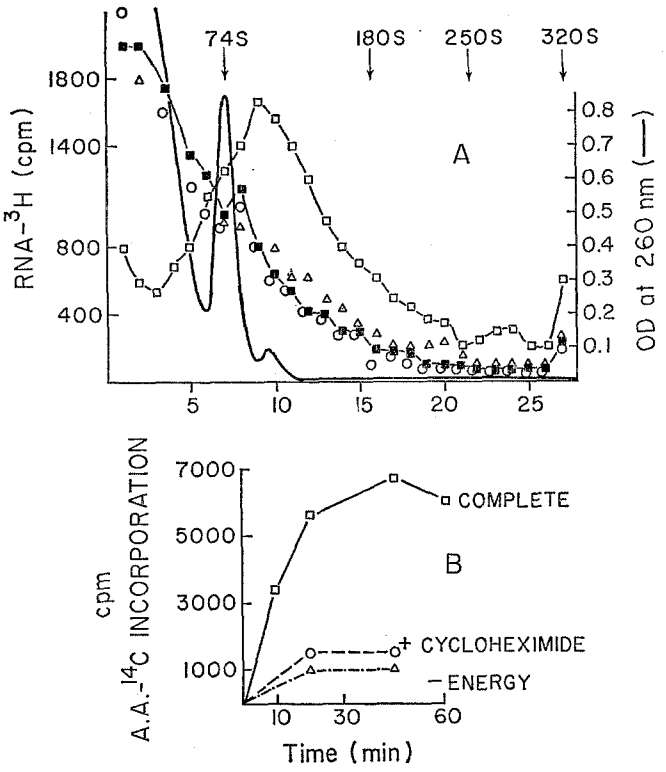

FIGURE 3. Breakdown of ^{3}H-labeled viral RNA ribosome complexes in the cell-free system, under conditions inhibitory to amino acid incorporation. *A*, 74S ribosomes from interferon-treated cells mixed with SFV ^{3}H-RNA and incubated with control cell sap in the complete cell-free system for 25 min. □—□, at 0°C; ■—■, at 37°C; ○—○, at 37° C in the absence of ATP, GTP, and phosphoenolpyruvate. *B*, Amino acid incorporation by a total ribosome preparation derived from control cells, assayed under the same conditions as those used in *A*.

would seem to be the most likely site of interferon action on general grounds. Studies in cell-free systems provided an obvious means of further analyzing the mechanisms of inhibition of viral protein synthesis. The objectives of these investigations have been to study the messenger function of viral RNA in cell-free systems using components from interferon-treated cells. However, the significance of the various parameters of messenger function that have been used in these studies is not clear. With respect to the binding of viral RNA to

ribosomes that we have observed and that was reported in the studies of Marcus and Salb (1966) and Carter and Levy (1967), there is no indication that this is related to the binding that occurs in the translation of a functional messenger RNA. We believe that there must be some doubt as to the validity of assessing translation by breakdown on incubation of RNA ribosome complexes formed in the cold. The inability of viral RNA to stimulate amino acid incorporation into polypeptides in a cell-free system when ribosomes from interferon-treated cells were used has also been reported (Marcus and Salb, 1966; Carter and Levy, 1967). However, unequivocal results in studies of this type are only possible if the viral RNA-directed incorporation can be shown to be meaningful, and this entails the identification of the product of the cell-free incubation as viral specific; in none of the studies reported has this condition been met.

In view of these considerations, we feel that there must be some doubt as to whether the ribosome is directly involved at all in the antiviral action of interferon. Even if there should be a ribosomal defect in interferon-treated cells, the participation of a newly synthesized TIP in the functional abnormality is highly speculative, and as I have discussed earlier, the proposal that cells respond to interferon by making a specific protein is itself also conjectural.

One possible resolution of the apparently conflicting results in this field would be if the alteration in the interferon-treated cell were to involve a factor or factors required for virus protein synthesis which is not an integral part of the ribosome, but which would be associated with it under some isolation procedures. Such a factor may for example be involved in the initiation of protein synthesis, or it may be a factor concerned with a necessary processing of the RNA before it is able to function as a messenger. Reservations regarding the role of a newly synthesized protein would of course apply to these suggestions as well as to the altered ribosome hypothesis.

That interferon induces the synthesis of a specific protein, and that ribosomes in interferon-treated cells have a specific functional abnormality, remain hypotheses. Putting these two hypotheses together has produced a third, that of the translational inhibitory protein. Attractive as they are, they remain speculations, and I hope they will be returned to their proper place as subjects of controversy.

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Discussion from the Floor

Dr. Levy (National Institutes of Health): In accord with the report of Dr. Sonnabend, we would agree thoroughly that the demonstration of differences in proteins in interferon-treated and control ribosomes is very difficult to establish. We looked at this perhaps not quite as often as you have, but almost as often, and we find no reproducible differences. As a matter of fact, the only reproducible differences that I thought I hopefully saw were in some earlier publications of yours which you feel is probably not really meaningful. So it may be that there aren't any.

Let's consider the question of binding of RNA to ribosomes. You used the sucrose gradient demonstration of binding which is the same which we used. It's something of a nuisance to perform this kind of technique, and one can't handle very many. Along with Dr. Dianzani this summer, we did some preliminary work with a different technique to demonstrate binding and possible differences in binding. We incubated ribosomes with RNA's, and then we put them through Millipore membranes. The single-stranded, viral RNA goes through a Millipore membrane quite well, while that which is bound to ribosomes would presumably be retained. I was away during the time Dr. Dianzani did the first experiments. I spoke with him on the telephone, and he was very excited. The interferon-type ribosomes bound normal cell RNA as well as control ones, but there was some 20-fold difference in the ability of the interferon-type ribosomes to bind viral RNA. We had the ribosomes in the freezer and did the experiment again about 10 days later. The differences were maybe threefold. We did the experiment again, and there were no differences. I'm not sure whether this means the differences were decaying. It could be that this is a rather sensitive kind of phenomenon. I agree with you, we have to hold the final decision in abeyance.

Dr. Armstrong: I also tried these binding experiments with Millipore filters using Sindbis virus RNA and rabbit ribosomes. And we found exceptionally good binding with the whole ribosome, with large subunits, with small subunits, and with the cell sap from interferon-treated cells or normal cells—it really didn't seem to matter, everything bound RNA to the same extent, sometimes 80–90%.