Effect of an Interferon on Synthesis of Viral Ribonucleic Acid and Plaque Formation.* (28090)

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The inhibitory properties of an interferon (S-VIF) obtained from fluids of chick cell cultures infected with Sindbis virus have been reported(1,2). Here we would like to expand on two previous findings: First, it was found that viral inhibition, as measured by reduction of plaque formation did not increase linearly with concentrations of interferon(2). Recently, however, reports of such a linear relationship have appeared(3,4). To investigate this discrepancy, it was decided to reexamine the dose response relationship of interferon using a preparation of greater potency which was effective in dilutions of 1:256 or higher.

Second, it was previously demonstrated that S-VIF interferon inhibited in chick cell cultures the infectivity of poliovirus ribonucleic acid (RNA) to about the same extent as it inhibited the infectivity of complete virus(1). This plus other considerations suggested that interferon acted intracellularly on virus synthesis or assembly. Similar data have been obtained by others(5). In addition, DeSomer *et al.* provided data showing that an interferon prevented the synthesis of cold phenol extractable infectious RNA of Western equine encephalitis (WEE) virus (6). Since this type of RNA represents a cell-associated viral component before its incorporation into intact infectious virus rather than being a derivative of such virus(7), its inhibition raised the possibility that the action of interferon consists in the inhibition of virus RNA synthesis. To test this hypothesis, syntheses of both infectious RNA and total virus were concurrently followed and compared in cultures pretreated with interferon and in controls.

Materials and methods. WS influenza A virus was kindly supplied by Dr. R. R. Wagner, and maintained by serial passages in the allantoic cavity of 11-day-old chick embryos. The source of EEE virus and method of preparing chick fibroblast cultures and media have been previously described(1,2). For extraction and titration of infectious RNA and to obtain samples to measure virus synthesis, chick cultures in 3 oz. bottles were prepared by inoculating 4 $\times$ the usual number of cells and used 48 hours after initiation, at which time the cell counts averaged 2.5×10^7 per culture.

WS-interferon. Preparations consisted of harvests of allantoic fluid heat inactivated at 56°C for 30 minutes 3 days after inoculation of 11-day-old chick embryos with 0.1 ml of 10^{-2} WS influenza stock virus. The biological activity of these preparations was comparable to those originally described by Wagner(8). No infectivity was detected in the lots of WS-interferon used in this work. Identically treated allantoic fluid harvests of uninfected embryos were used as "control fluids." These did not affect virus infectivity. The method of titrating interferon was as described(2). Inhibition of plaque formation in treated cultures was expressed in percentage of plaques with respect to input virus (2).

Extraction and titration of infectious RNA. By RNA, we mean cell-associated infectious RNA of EEE virus as titrated biologically and characterized by Wecker(7,9). RNA was prepared as follows: Monolayers of chick cells consisting of about 2.5×10^7 cells were each incubated at room temperature (25-26°) for 18 hours with 0.3 ml of 1:5 dilution of WS-interferon or 1:5 dilution of control allantoic fluid. Before infection, the interferon was washed off the cultures with 5 ml Hanks' BSS. They were then each inoculated with 0.1 ml of serum free stock EEE virus at input multiplicities of 10 to 100. To permit adsorption of virus, the cultures were incubated at 36°C for 1 hour.

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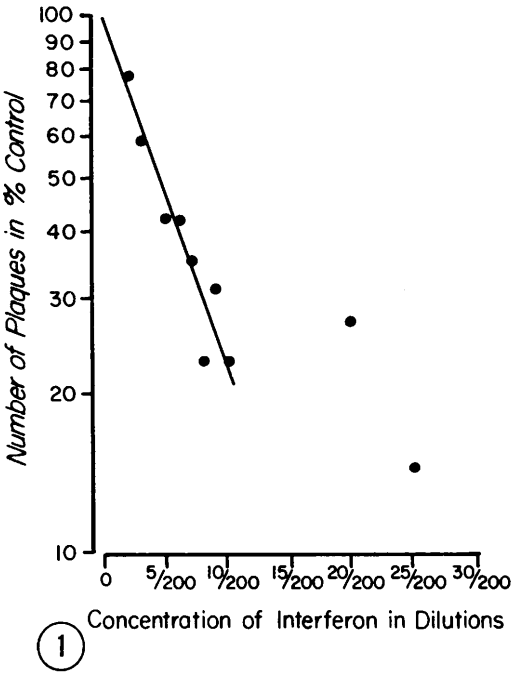
Then they were decanted and washed with 10 ml prewarmed Hanks' BSS. To each culture 5 ml Eagle's basal amino acid and vitamin mixture in Earle's BSS (EE) was added before incubation at 36°C. For RNA extraction, Wecker's method(9) was followed with minor modifications: Cells from 5 identically treated cultures were mechanically detached, pooled and suspended in 10 ml of culture fluid. The pooled cells were then sedimented at 1000 r.p.m. for 10 minutes, suspended in 4 ml EE and stored frozen at −70°C. The suspension was extracted twice with 80% phenol and the RNA was dissolved in 3 ml distilled water after alcohol precipitation. For titration of RNA, 0.2 ml aliquots of dilutions made in M $MgSO_4$ were inoculated in monolayers of concentrated chick cells pretreated as described(9). To an initial 7 ml agar overlay without neutral red a second 7 ml overlay with the dye was added after 2 days of incubation. Plaques were counted one day thereafter.

Virus assays. One ml samples were obtained from the pool of detached cells in 10 ml culture medium. These were frozen and thawed once to release cellular virus. After sedimentation of cell debris at 1000 r.p.m. for 5 minutes the supernatants were titrated by plaque formation using 0.1 ml inocula.

Results. Effect of WS-interferon on plaque reduction. Experiments were undertaken to determine the optimum duration of preincubation with interferon for maximal inhibitory effect. In one of these, it was found that upon challenge, a culture preincubated with 0.3 ml of 1:100 interferon for 3 hours at 37°C showed 151 plaques while a comparable culture preincubated for 18 hours at 25-26°C showed 173. These values represented respectively 69% and 77% of input EEE virus. These results suggested that prolonged incubation for 18 hours under these circumstances did not produce more inhibition than did incubation for 3 hours.

To test the dose response relationship: Twenty cultures were incubated for 3 hours at 36°C with dilutions of interferon indicated in Fig. 1, washed and inoculated with challenge EEE virus. Each point in the Figure represents the mean of plaque counts of duplicate treated cultures divided by input virus as determined in 2 control titra-



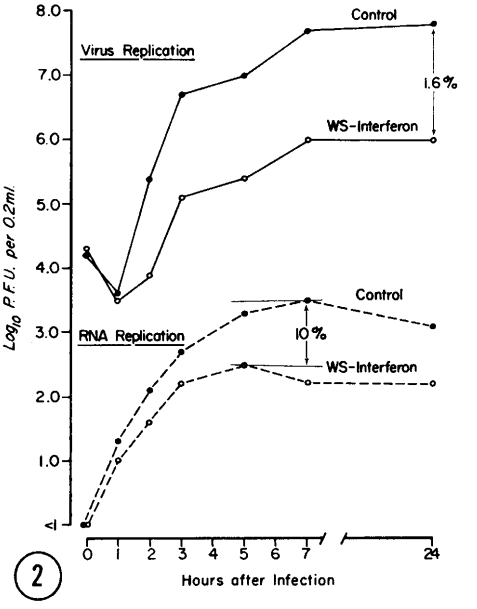


FIG. 1. Effect of concentration of WS-interferon on its plaque reducing capacity.

FIG. 2. Viral RNA and infectious virus replication in chick cultures pretreated with WS-interferon and in controls. These cultures were inoculated at a high multiplicity with EEE, and viral RNA and virus output were assayed. Points represent mean of 2 identical experiments (see text).

tions. A straight line was fitted through the 8 experimental points between interferon dilutions 3:200 and 1:20 *and* through the "origin" defined by 0 interferon concentration and 100% of control plaque number. The equation of this line was found to be $y = 2 - 13.0\ x$, where $y = \log 100\ P/P_o$, $100\ P/P_o$ being the vertical plot in Fig. 2, representing number of plaques counted in treated cultures (P) in per cent of number of control counts (P_o), and x the dose of interferon in dilutions. On the other hand, the ordinary regression line based only on the 8 observed points without requiring it to pass through the "origin" (0, 100%) was found to be $y = 2.04 - 14.13\ x$. This line was very close to the one obtained before. The standard deviation of the slope (14.13) was 2.14, and the linearity was highly significant. The slope of the former line (13.0) was within half a standard deviation of the slope of the present one. The actual curve may then be assumed to be linear and to pass through the origin. This relationship is consistent with single hit kinetics, in which one unit of interferon, presumably one molecule, is sufficient to render a cell resistant.

However two points representing the effect of interferon at dilutions 1:10 and 1:8 could not be incorporated in the regression line, confirming the earlier finding that inhibition at higher concentration of interferon is nonlinear(5). Determinations of plaque reduction with interferon at dilution of interferon lower than this could not be consistently carried out because higher viral inocula (>1000 P.F.U.) frequently produced streaky cultures with uncountable numbers of plaques, while plaque formation using smaller inocula was completely inhibited.

In summary, it appears from the above data that 1) in accordance with our previous findings(2,5) viral inhibition by interferon does not increase linearly with respect to wide dosage ranges, but 2) a linear relationship can be demonstrated in the lower range.

Effect of WS-interferon on viral RNA and virus synthesis. To obtain a valid comparison of the inhibition of RNA and virus synthesis for the purpose of ascertaining whether inhibition of RNA can account for inhibition of viral replication, the following experimental conditions were met: 1) high multiplicities of infection were used in interferon treated and control cultures to minimize multiple infectious cycles and secondary elaboration of interferon(2); 2) the dilution of interferon used (1:5) consistently inhibited both RNA and virus output but permitted enough replication so that both moieties could be measured; 3) to follow the variation of inhibition and to obtain maximal titers of virus and RNA for comparison, the time course of virus and RNA replication was followed.

Seven groups each consisting of 5 chick cultures were pretreated with 1:5 control allantoic fluid and 7 similar groups were incubated with 1:5 WS-interferon for 18 hours at 25-26°C. The 70 cultures were then washed and inoculated with EEE virus at an input multiplicity of 40. This was allowed to adsorb at 36°C for 1 hour. Immediately and 1, 2, 3, 5, 7 and 24 hours after infection, one control and one treated group of cultures were collected and processed for extraction of infectious RNA and collection of virus samples. The extracted material and virus samples were then titrated by plaque formation on chick cultures. A duplicate experiment also involving 70 cultures was subsequently performed. The mean titers of infectious RNA and total virus from these 2 experiments were calculated on the basis of 0.2 ml inoculum and graphed in Fig. 2.

Considering the course of infectious virus and RNA replication in controls: The synthesis of viral RNA was evident 1 hour after infection or end of adsorption period, when there was evidence of viral eclipse, and was completed after 5-7 hours, or earlier than that of infectious virus. The ratios of RNA to infectious virus titers in *controls* at 1 and 2 hours were respectively 1:200 and 1:2,000; while comparable ratios at 7 and 24 hours were 1:15,900 and 1:50,100. The lack of parallelism between the titer of RNA and of infectious virus is what one would expect if the RNA extracted were not derived from intact infectious virus but represented a cell associated species which gradually became un-

available by cold phenol extraction as it was incorporated into intact virus(7,9).

Comparing now the synthesis of RNA and infectious virus in interferon-treated cultures and in controls: Both moieties were reduced in relation to controls after 1 hour of infection and remained so throughout the infection. At 2 hours after infection, RNA extracted from treated cultures was 32% that of controls, while total virus was 3.2% of that of controls. At 3 hours after infection, treated cultures yielded 32% as much RNA as controls, and 2.5% as much virus. At 5, 7 and 24 hours, treated cultures yielded 16%, 5% and 13% as much RNA as controls, and 2.5%, 2% and 1.6% as much virus. It is apparent that at each of these different time periods, the inhibition of RNA lags behind that of infective virus. Therefore, infective virus was inhibited to a greater degree than infectious RNA.

The same conclusion may be reached by observing peak values of RNA and virus synthesis. The peak RNA titer of control cultures was $10^{3.5}$ while that of treated cultures was $10^{2.5}$ P.F.U. per 0.2 ml. Peak RNA synthesis in treated cultures was therefore 10% of control values. Peak total virus titer of treated cultures was $10^{6.0}$ P.F.U. per 0.2 ml, representing 1.6% of the peak control titer, which was $10^{7.8}$ P.F.U. per 0.2 ml. The ratio of peak RNA to infective virus in treated cultures was 1:3,170; a comparable ratio in control culture was $10^{3.5}$ to $10^{7.8}$ or 1:20,000.

Two additional experiments following the general outline of the experiment described above produced comparable results. That is, both RNA and virus syntheses were inhibited, but inhibition of virus was consistently greater.

Discussion. While it is well known that interferon inhibits virus replication rather than virus penetration or uncoupling(2), it is not known whether it specifically inhibits the synthesis of a particular viral component or the assembly of these components. The finding that the synthesis of infectious, cell-associated RNA of WEE virus(6) as well as EEE virus is inhibited by interferon raised the possibility that viral RNA inhibition may be the basis of its action, despite the fact that the thesis that interferon acts by inhibiting RNA in general had been previously adopted and subsequently abandoned by Isaacs on biochemical grounds(5).

However, our findings described herein that interferon inhibits synthesis of infective virus to a greater degree than viral RNA, suggest that interferon does not act by only specifically inhibiting viral RNA synthesis. Other components of virus, such as proteins or lipids, may also be affected or some reaction underlying the assembly of these components is impaired. It is also possible that infectious RNA is extracted more efficiently from interferon-treated cultures than from control cultures because of formation of defective virus. Admittedly one might assume that in view of the interdependence between viral RNA and protein synthesis(9), the activity of interferon could still be explained on the basis of specific inhibiton of viral RNA. Thus, the greater inhibition of infective virus could be due to decreased protein synthesis secondary to deficient RNA template material. However, on the basis that interferon acts on energy metabolism(5), and that it may possess a non-specific cell depressing effect(10), it appears more reasonable to postulate that more than one reaction in virus synthesis is inhibited by it.

These and certain other characteristics of the action of interferon also help explain our finding that inhibition by interferon does not increase linearly with respect to all concentrations of interferon. In addition to the cell depressing effect of interferon, its effect may be overwhelmed by large doses of virus(2,11). Furthermore, it has been recently found that interferons prepared from certain Group A Arboviruses are more effective when diluted (12), suggesting that there may be potentiators of infection in undiluted crude preparations. Such data suggest that there may be more than one site or type of cell-interferon reaction. They may partially account for the deviation from linearity of the dose-response curve. Clarification of this point will require further work using purified interferon preparations, and characterization of possible potentiators of infection.

Summary. An interferon prepared from

the allantoic fluid of chick embryos infected with WS influenza A was found to inhibit plaque formation of EEE virus linearly in a semi-logarithmic plot between dilutions of 3:200 and 1:20. Higher concentrations of interferon, however, did not inhibit plaque formation in the predicated manner. This interferon also inhibited synthesis of cell-associated viral RNA in chick cultures infected with EEE virus. However, such inhibition was found to be consistently less than the inhibition of infectious virus replication. It is suggested that interferon does not act merely by inhibtion of viral RNA but that it interferes either directly or indirectly with the synthesis of other viral components or with the reactions underlying the assembly of these components.

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Chromatographic Separation of Glucagon and Insulin from Serum By Resin-Exchange Paper* (28091)

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During an attempt to establish a practical method for quantitative assay of glucagon in mammalian serum, a simple, easy approach to separating the hormone and coincidentally insulin from serum was evolved. This method employs descending chromatography on a specially prepared resin-exchange paper. In all commercially available resin-impregnated papers previously employed chromatographically, both hormones remain at the site of application, as does the serum. By use of a special resin-exchange paper, 30% Amberlite produced by Rohm and Haas of Philadelphia, both hormones mechanically mixed with the serum migrate independently at constant rates out of the serum into the field from the point of application.

Materials. 1. Resin-exchange paper, 30% impregnated Amberlite, IRC 50 (W.A. 1) of Rohm and Haas.† The paper was cut into strips ¾″ × 8″, each strip being pencil-marked for subsequent point of application. Before applying any test material the paper was washed for one-half hour in absolute ethanol and dried under a heat lamp. Unless this preliminary step is taken, neither glucagon nor insulin will leave the site of its application.

2. Standard chromatography chambers.

Solutions. 1. Stock solution of glucagon: to a beaker containing 10 mg of crystalline

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