Chemical analyses were satisfactory for the pyrrolidine itself, an *N*-phenylurea derivative (obtained in 92.5 percent yield), and for an oxalic acid salt as well. Hence I believe that the minor component is the *trans* isomer.

The pyrrolidine exhibited hemolytic properties when tested with human, chicken, and pigeon blood. Solenamine was also hemolytic in a test with rabbit blood (*2*). However, residue tests on houseflies, *Musca domestica* (L.), both DTT-susceptible and -resistant, showed no insecticidal action, although fire ant venom is reported to be active against this species of insect. Adrouny, by means of a gas-chromatographic comparison of my material with solenamine, demonstrated that 2-methyl-3-hecadecylpyrrolidine (*cis* or *trans*) is not a component of the insect extract, solenamine. The retention time of one of the components of solenamine is quite close to the peaks of that of the pyrrolidine mixture; the 3-pyrroline, or a structure closely related to these, may yet be correct.

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3 May 1967

Interferon Binding: The First Step in Establishment of Antiviral Activity

Abstract. *Chick cells incubated at 1°C with interferon fail to develop antiviral activity, but this activity appears subsequent to a 7-hour incubation at 37°C after removal of interferon by repeated washings. Treatment with actinomycin D blocks the development of the latter activity. Cells incubated with interferon at 1°C for up to 1 hour and then washed and incubated for 2 hours at 37°C develop a degree of antiviral activity proportional to the concentration of interferon at initial incubation; at any concentration, the antiviral activity increased with the duration of initial incubation at 1°C, but a maximal response was reached at 10 or 20 minutes. Treatment with trypsin after incubation with interferon at 1°C inhibited development of antiviral activity. Interferon is rapidly bound to a superficial cell site, and this binding is necessary for development of antiviral activity in chick cells.*

The induction of antiviral activity in cells by interferon is a complex process. The first step takes place at 1°C; the second step, which apparently involves RNA and protein synthesis, requires that cells treated with interferon be incubated for some time at 37°C before virus infection (*1*). I studied the first step. When incubated with interferon at 1°C and then washed, the cells developed resistance to viral infection after incubation at 37°C for 1 hour or more. Because there is no detectable uptake of interferon by cells during the initial incubation period (*2*), interferon possibly initiates a series of reactions which lead to antiviral activity but does not itself bind to cells. My studies show, however, that the development of antiviral activity subsequent to exposure of cells to interferon at 1°C requires the binding of interferon to a superficial cell site.

Monolayers of chick embryo fibroblasts (CEF) were prepared (*3*). Semliki forest virus (SFV) was prepared in CEF treated with actinomycin D. Plaque assays of SFV titers were performed on CEF (*3*). Each milliliter of the solution of partially purified interferon contained 10,000 units of interferon and 179 μg of protein, a unit being the amount which inhibited development of SFV plaques by 50 percent (*4*).

The CEF monolayers were incubated with or without 3 units of interferon per milliliter for 4 hours at 1°C or 37°C and then washed six times with cold Eagle's medium. Some monolayers incubated at 1°C or 37°C were immediately infected with SFV at a virus : cell multiplicity of 40 : 1. The virus was adsorbed at 37°C for 1 hour; the cells were washed six times and incubated in Eagle's medium with 10 percent calf serum for 7 hours, at which time the log phase of virus growth ended. The monolayers and fluids were then frozen, thawed, and assayed for virus. No antiviral activity was present in cells kept at 1°C (Table 1), while those at 37°C had developed strong antiviral activity.

In the same experiment, other monolayers incubated at 1°C or 37°C with or without interferon for 4 hours were washed and then incubated for an additional 7 hours at 37°C. The cells were then infected and treated as in the other group. In the cells incubated with interferon at 1°C, the antiviral activity was approximately as strong as that in other cells incubated with interferon for 4 hours at 37°C (Table 1). Cells incubated with actinomycin D fail to develop antiviral activity when subsequently incubated with interferon (*3*). When actinomycin D was added to cells incubated with interferon at 1°C and then washed, no antiviral activity developed (Table 1). My results confirmed those of other workers who showed that after incubation of cells with interferon at 4°C a period of incubation at 37°C was required for development of antiviral activity (*5*). In addition, my findings showed that, whatever interferon-cell interaction had taken place in the cold, the development of antiviral activity could be blocked by actinomycin D.

The nature of the cell-interferon interaction at 1°C was further delineated by the following experiments. Various concentrations of interferon were incubated with CEF at 1°C for 1 to 60 minutes. The cells were washed 6 times with 10 ml of cold medium, incubated at 37°C for 2 hours, and then infected with SFV. No antiviral activity was present in the tissue cultures after the 2-hour incubation at 37°C; this indicated that no release of previously

Table 1. Cells infected with Semliki forest virus immediately after a 4-hour incubation at 1°C or 37°C or after an additional 7-hour incubation at 37°C.

Treatment and initial incubation temperature	Virus titer	
	Initial incubation	Additional 7 hours at 37°C
None (37°C)	17×10^6	20×10^6
Interferon, 3 units (37°C)	1.8×10^6	1.2×10^6
None (1°C)	13×10^6	14×10^6
Interferon, 3 units (1°C)	10×10^6	2.0×10^6
None (1°C) + Act. D*		15×10^6
Interferon, 3 units (1°C) + Act. D		14×10^6

* Actinomycin D (Act. D) (2 μg/ml) was added at the end of the initial incubation.

bound interferon occurred. Assays of virus growth in the various cultures showed that the degree of virus inhibition was proportional to the concentration of interferon (Fig. 1). At any concentration, the response increased with the duration of exposure to interferon at 1°C, but in all cases a maximum response was reached by 20 minutes, and in cells treated with 1000 units of interferon per milliliter it was reached by 10 minutes. Incubation thereafter for up to 60 minutes (up to 120 minutes in other experiments) did not increase the degree of virus inhibition observed. This suggested that an equilibrium reaction involving interferon and a cell binding site might be taking place at 1°C.

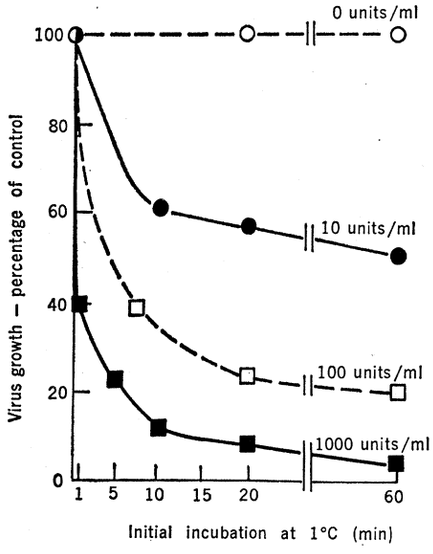

Fig. 1. Interferon-cell interaction at 1°C. Chick cells were incubated with various concentrations of interferon for the indicated periods of time, washed six times with cold medium, incubated at 37°C for 2 hours, and then infected with virus. The virus titer was assayed on chick monolayers by a plaque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

To decide whether this reaction required binding of interferon, I attempted to remove any interferon present after the incubation at 1°C. Interferon is readily destroyed by trypsin. Cells were therefore sequentially (i) incubated with 1000 units of interferon per milliliter for 20 minutes at 1°C; (ii) washed six times to remove interferon; (iii) treated with 3 mg of trypsin per milliliter at 1°C for 10 minutes; (iv) treated with an excess of soybean trypsin inhibitor (6 mg/ml); (v) washed six times with cold medium containing 10 percent serum; and (vi) incubated for 2 hours at 37°C with this medium. After this incubation period the cells were infected, the virus was harvested after 7 hours, and virus titers were assayed. The results (Table 2) indicated that treatment with trypsin inhibited the subsequent development of antiviral activity. Cells treated with trypsin alone had somewhat poorer virus yields than untreated controls had, probably due to cell damage caused by trypsin treatment. However, this treatment did not damage cells in such a manner that antiviral activity could not develop, as shown by the fact that cells treated with trypsin at 3 mg/ml for 10 minutes before incubation with interferon developed full antiviral activity (Table 2). Also, full antiviral activity remained in cells treated with trypsin after sufficient incubation at 37°C with interferon to develop marked antiviral activity.

At 1°C interferon binds rapidly and firmly to superficial sites on the cells. This binding is necessary for development of antiviral activity because the bound interferon can be destroyed by trypsin, resulting in failure to develop antiviral activity. Studies to elucidate the nature of the binding site have indicated that it is sensitive to the action of a purified enzyme, phospholipase C.

The binding of interferon to a particular site must be quite firm, as bound interferon was not removed from chick cells by repeated washing and did not appear in culture fluids after incubation for 2 hours at 37°C with cells treated previously with interferon. The amount of interferon bound was only a small portion of the interferon applied, however, in view of the fact that assay of a solution of interferon after incubation for 20 minutes with CEF at 1°C showed no significant loss of antiviral activity from the solution. I have been able to extract very small amounts of antiviral activity (< 5 units) from washed cells preincubated with 1000 units of interferon per milliliter as Levine did (*5*). It is uncertain what portion, if any, of the extracted antiviral activity represents interferon involved in an interferon-cell interaction which specifically results in the development of cellular antiviral activity.

Table 2. Development of antiviral activity by trypsin treatment before or after initial incubation with interferon at 1°C.

Treatment	Virus titer	
None	11×10^6	18×10^6
Interferon, 1000 unit/ml	1.6×10^6	3.0×10^6
Trypsin, 3 mg/ml (1°C)	4.4×10^6	5.7×10^6
Trypsin after interferon	4.0×10^6	
Interferon after trypsin		1.2×10^6

My results were similar to those found in studies of the mechanism of action of colicins (*6*) and the polypeptide hormones, thyroid-stimulating hormone, insulin (*7*), and ACTH (*8*). In these systems, rapid binding of colicin or hormone took place, and the activity of bound colicin or hormone could be destroyed by incubation with trypsin (*6, 7*) or with specific antiserum (*7*). Thus, some similarities apparently exist in the steps necessary for the initiation of biological activity by colicins, polypeptide hormones, and interferon because all seem to involve binding to a superficial cell site.

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