

Interferon Production and Protein Synthesis in Chick Cells

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ABSTRACT

FRIEDMAN, ROBERT M. (National Cancer Institute, Bethesda, Md.). Interferon production and protein synthesis in chick cells. *J. Bacteriol.* 91:1224-1229. 1966.— Overnight incubation of chick embryo fibroblasts (CEF) at 4 C before infection with live Semliki Forest virus (SFV) increased virus yields but decreased interferon production. The same findings were noted when CEF were incubated for 4 hr with *p*-fluorophenylalanine (FPA) before infection with live SFV or inactivated Chikungunya virus. In both systems incorporation of C¹⁴-leucine into protein appeared to be increased after pretreatment at 4 C or with FPA. Protein synthesis could be raised in CEF incubated in 0.5% serum after trypsinization by increasing the concentration of serum. CEF in 10% serum had higher rates of C¹⁴-leucine incorporation than did cells in 1.5% serum, but again the cells with the apparently high rate of incorporation produced less interferon. These findings may be related to the mechanism of cellular control over interferon production.

Studies on the antiviral protein interferon (11) have shown that its production is induced by infection of cells with live or inactivated ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) viruses (9) and, possibly, by nonviral substances (10, 12, 16, 19, 20). The formation of interferon induced by an RNA virus was blocked by actinomycin D treatment of cells before their infection (8), or by inhibitors of protein synthesis (9). These findings suggest, respectively, that the information for interferon synthesis is coded in the genome of the cell and that production of interferon probably requires *de novo* protein synthesis.

In the present study, it was found that three sets of experimental conditions which appeared to raise the level of protein synthesis in primary chick embryo fibroblast (CEF) cultures also tended to decrease the subsequent ability of these cultures to produce interferon. These findings are of interest because of their possible relationship to cellular control over interferon production.

MATERIALS AND METHODS

Cells and viruses. The preparation of CEF monolayers and the media used have been previously described (18). Under these conditions, the number of cells in the confluent monolayers employed was stable with all of the experimental conditions used.

Mouse brain pools of the Kumba strain of Semliki Forest virus (SFV) and of Chikungunya virus were employed. Chikungunya virus was heat-inactivated at 36 C for 23 hr (7).

Virus assays. The plaque assay for SFV has been previously described (4). Final plaque counts were made 72 hr after infection of monolayers with virus.

Interferon assay. CEF monolayers were assayed for interferon by the vaccinia plaque reduction method of Lindemann and Gifford (13). The titer (in units) was the reciprocal of that tissue fluid dilution which reduced the number of vaccinia virus plaques by 50%. Tissue fluids were heat-inactivated at 65 C for 30 min before being assayed for interferon. This treatment destroyed the capacity of arboviruses to induce interferon formation.

Amino acid incorporation. Incorporation of C¹⁴-leucine was studied by one of two methods. In some studies, the cover-slip method of Baltimore and Franklin (3) done in six or eight replicates for each determination was employed; in others, the cells were washed with cold medium containing leucine, removed from glass with ethylenediaminetetraacetate, and precipitated with 7% cold trichloroacetic acid. These precipitates were extracted twice with hot (90 C) trichloroacetic acid and then with chloroform-ether-alcohol (1:2:2) at 37 C. The precipitates were suspended in 0.8 ml of dry ether, quickly plated on planchets, and counted in a windowless gas-flow counter. Specimens were analyzed in triplicate, each specimen containing 5×10^7 cells. Cell counts were made on replicates of each group of cells employed.

Multiple determinations, all in substantial agreement with the data presented, were made.

Reagents. DL-*p*-Fluorophenylalanine (FPA) and DL-phenylalanine were purchased from Calbiochem. DL- C^{14} -Leucine (36.4 mc/mmmole) was obtained from The Radiochemical Centre, Amersham, England.

RESULTS

Effect of preincubation of cells at 4 C. During the course of studies on the action of interferon, it was noted that cells which had been incubated overnight at 4 C often supported the growth of SFV better than did controls incubated at 37 C. Fully sheeted CEF monolayers containing 2.5×10^7 cells were incubated overnight at 4 or 36 C. Cells which had been kept at 4 C were transferred to 36 C for 1 hr, and then both sets of plates were infected with SFV at a virus-to-cell multiplicity of 0.01. At the times indicated in Fig. 1, plates were removed from the 36 C incubator and placed at -70 C. After freezing, the plates were thawed, and the fluids were assayed for virus and for interferon.

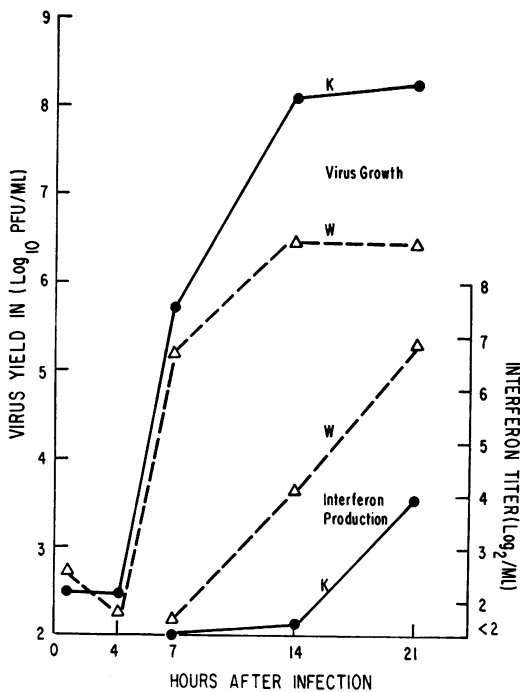


FIG. 1. Effect of 4 C pretreatment on production of Semliki Forest virus (SFV) and interferon in chick embryo fibroblast monolayers (CEF). CEF were incubated overnight at 4 C (K) or 36 C (W), washed, warmed, and then infected with SFV at a virus-to-cell multiplicity of 0.01. Virus growth was halted by freezing at indicated times and fluids were assayed for interferon and virus.

The cells which had been kept at 4 C supported the growth of virus better than did those kept at 36 C (Fig. 1); however, the cells which had been kept at 36 C were better producers of interferon. This suggested that the increased virus yield in cells which had been incubated at 4 C may have been related to the decrease in the endogenous production of interferon (8).

Effect of preincubation of cells with FPA on interferon production. While studying the inhibition of interferon action caused by FPA (5), an effect similar in some respects to that just described was noted. CEF were incubated for 4 hr with 100 μ g/ml of FPA in balanced salt solution buffered with 0.002 M tris(hydroxymethylamino)methane (Tris). The cells were washed five times to remove FPA and were then infected with SFV at virus-to-cell multiplicities of 0.01 or 2.0. Virus growth medium consisted of Tris-buffered salts supplemented with 0.25% lactalbumin hydrolysate and 100 μ g/ml of phenylalanine. Virus yields and interferon production at 14 and 21 hr after infection were measured as in the previously described experiment and are listed in Table 1. It was noted that in cells infected with a low multiplicity of virus, pretreatment with FPA increased the yield of virus but reduced the production of interferon (Table 1, line A); however, in cells infected with a higher multiplicity of virus, this

TABLE 1. Virus yield and interferon production in *p*-fluorophenylalanine (FPA)-pretreated cells

Pretreatment*	Virus-cell multiplicity	Virus yield (plaques/ml $\times 10^6$) at		Interferon produced (units) at	
		14 hr	21 hr	14 hr	21 hr
(A) None FPA	0.01	1	2.5	20	210
		7	26	5	20
(B) None FPA	2	86	84	76	230
		69	83	57	380
(C) None FPA	Inactivated virus	—	—	40	160
		—	—	<4	20

* Chick embryo fibroblasts (24×10^6) were treated with 100 μ g/ml of FPA for 4 hr in Tris-buffered salts or in buffered salts alone. The cells were washed and then infected with Semliki Forest virus at the noted virus-cell multiplicity. Growth medium consisted of Tris-buffered salts, 0.25% lactalbumin, and 100 μ g/ml of DL-phenylalanine. After 14 or 21 hr of incubation, the plates were frozen and thawed, and the tissue culture fluids were assayed for virus and interferon production.

effect was not seen, and no significant differences in virus or interferon yield were noted (Table 1, line B).

In addition, an experiment was performed to test the effect of FPA pretreatment on the ability of cells to produce interferon when stimulated by an inactivated virus. The same conditions as in the previously described experiments were followed, but heat-inactivated Chikungunya virus was employed instead of active SFV. Again a decreased ability to produce interferon was observed in the FPA-pretreated cells (Table 1, line C).

To test whether the effect noted was due to FPA acting as an antimetabolite, heat-inactivated Chikungunya virus was again employed to stimulate interferon production. In this experiment (Fig. 2), cells were treated with FPA, FPA and phenylalanine simultaneously, or actinomycin D for 4 hr, washed, and infected with virus in medium containing phenylalanine. After 20 hr, tissue fluids were harvested, heated to 65 C for 30 min, and assayed for interferon. Again, the cells pretreated with FPA produced less interferon, as did negative control cells pretreated with actinomycin D (8); however, when phenylalanine was present with FPA, no effect of the FPA on the subsequent ability of cells to produce interferon was seen.

Treatment of cells with FPA before infection, or incubation at 4 C before infection, decreased the ability of the cells to make interferon. It was of interest to investigate protein synthesis in these cells.

Effect of treatment with FPA or incubation at

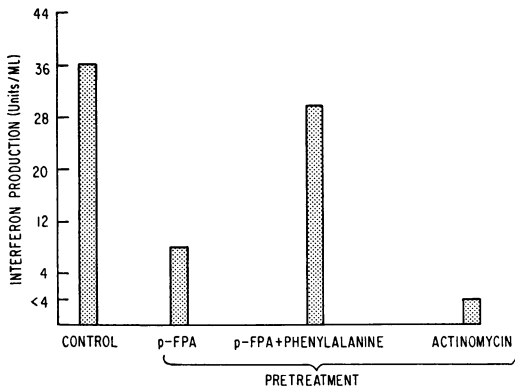


FIG. 2. Interferon production in chick embryo fibroblast monolayers (CEF) by heat-inactivated Chikungunya virus. CEF were pretreated for 4 hr with *p*-fluorophenylalanine (FPA, 100 μ g/ml), FPA + phenylalanine (both 100 μ g/ml), or actinomycin D (1 μ g/ml), washed, infected for 1 hr with heat-inactivated Chikungunya virus, and then allowed to incubate for 20 hr in the presence of 100 μ g/ml of phenylalanine. Fluids were heat-inactivated at 65 C and assayed for interferon.

4 C on cell protein synthesis. CEF monolayers containing 15×10^6 to 18×10^6 cells were treated with FPA for 4 hr or placed at 4 C overnight, as previously described. After washing off FPA or replacement at 36 C, the cells were incubated with the appropriate medium containing 0.1 μ C of C^{14} -leucine or were pulsed for 1 hr with 1 μ C of C^{14} -leucine. The results of studies on the incorporation of C^{14} -leucine into protein are shown in Fig. 3 and 4.

In Fig. 3, the effect of pretreatment with FPA appears to be a stimulation of protein synthesis as compared with controls, rather than a depression. In both the pulse label experiment (Fig. 3B) and the cumulative incorporation study (Fig. 3A), the number of counts was higher in the FPA-pretreated cells. The infection of cells with low multiplicities of virus or with heat-inactivated virus did not modify this finding, but in each of these cases the C^{14} -leucine incorporation was altered somewhat. For instance, in the case of infection with heat-inactivated Chikungunya virus, the rate of protein synthesis was increased over uninfected controls by about 30%, but the preincubation of cells with FPA still resulted in an additional increase comparable to that shown

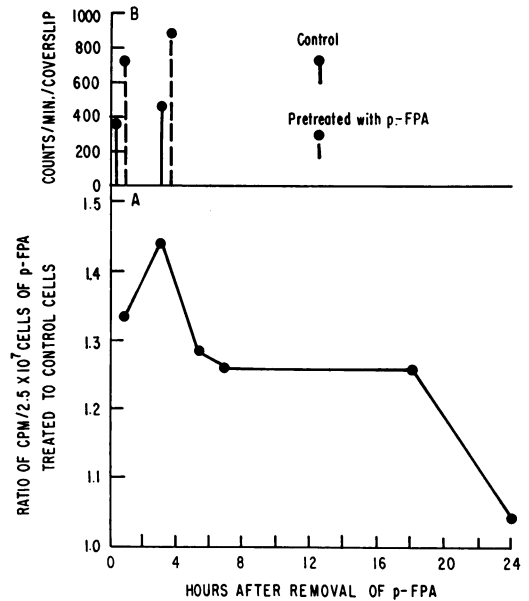


FIG. 3. Effect of pretreatment of CEF with *p*-fluorophenylalanine (FPA) on cell protein synthesis. CEF were pretreated with FPA for 4 hr, washed, and then either (A) 0.1 μ C of C^{14} -leucine was added and cells were harvested at indicated times, or (B) cover slips with cells were pulsed with 1.0 μ C of C^{14} -leucine for 1 hr at indicated times. In both experiments, the cells were then extracted for protein, and C^{14} incorporation was estimated (see Materials and Methods).

in Fig. 3. No effect on RNA synthesis had been noted after a 4-hr treatment with FPA (5).

Presented in Fig. 4 are results of experiments performed on cells incubated at 4 C and then allowed to incubate with C^{14} -leucine. Again, the cells preincubated at 4 C appeared to show an increase in amino acid incorporation as compared with controls in both pulse and cumulative incorporation studies.

It appeared, therefore, that in CEF pretreated with FPA or treated at 4 C the decreased ability to produce interferon was correlated, not with a decrease in the general ability to synthesize protein, but, if anything, with a stimulation of protein synthesis. An additional system was sought to investigate further these observations.

Effect of serum concentration on protein and interferon synthesis. Amos has shown that the level of protein synthesis in CEF which had been trypsinized and then incubated in medium with a low (0.5% or less) serum concentration could be increased by raising the percentage of serum in the medium. The level of protein synthesis increased directly with the new concentration of serum (1; Amos, *personal communication*).

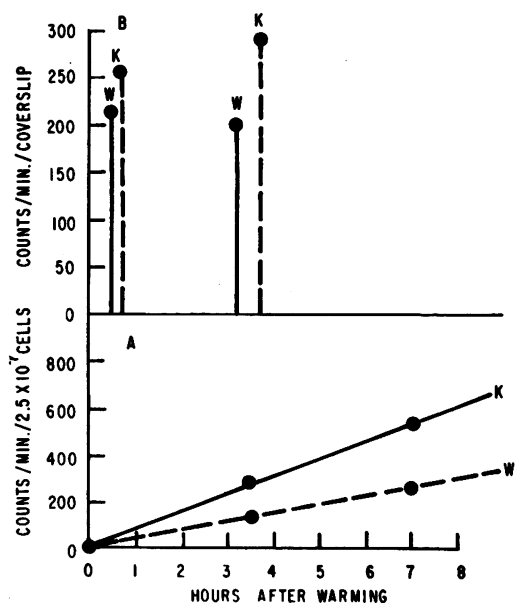


FIG. 4. Effect of 4 C pretreatment on cell protein synthesis. CEF were incubated overnight at 4 C (K) or 36 C (W), allowed to warm to 36 C, and then either (A) 0.1 μ c of C^{14} -leucine was added and cells were harvested at indicated times, or (B) cells on cover slips were pulsed with 1.0 μ c of C^{14} -leucine for 1 hr at the indicated times. In both experiments, the cells were then extracted for protein, and C^{14} incorporation was estimated (see Materials and Methods).

Figure 5 shows the results of an experiment performed under Amos' conditions. In confirmation of his findings, it was noted that, when the serum concentration of the medium of cells which had been kept in 0.5% serum for 36 hr after trypsinization was raised to 1.5 or to 10%, a rapid increase in the rate of C^{14} -leucine incorporation occurred, and that the cells shifted to 10% serum had the higher rate of incorporation under the new conditions.

When the cells were stimulated to produce interferon by use of heat-inactivated Chikungunya virus, it was again noted that those cells which had the higher rate of leucine incorporation produced less interferon (Fig. 5). Under the conditions of this study, the incorporation of

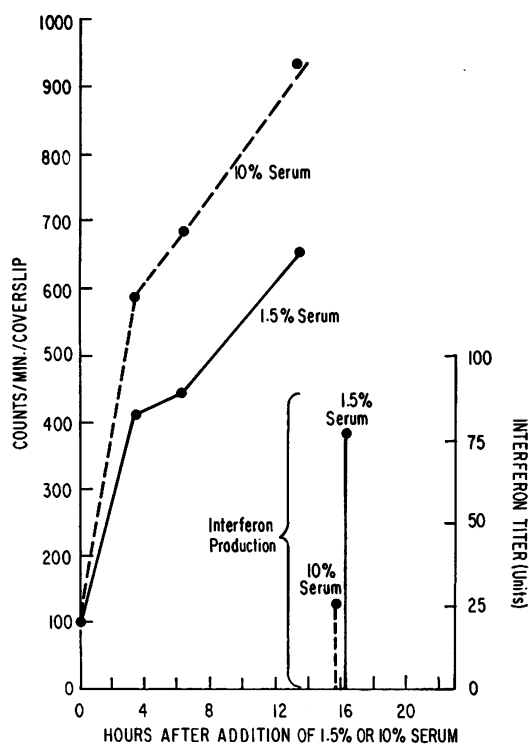


FIG. 5. Effect of serum concentration on protein synthesis and interferon production in chick embryo fibroblasts (CEF). CEF were trypsinized, and then suspended and allowed to attach to glass cover slips in 0.5% serum. After 36 hr, the serum concentrations were changed to 1.5 or 10%, and 1-hr pulses of C^{14} -leucine (1 μ c) were added at the indicated times. Cover slips were then extracted for protein, and C^{14} incorporation was estimated. Also, CEF monolayers trypsinized and treated with various concentrations of serum as described above were stimulated to produce interferon by heat-inactivated Chikungunya virus. After 16 hr of incubation, interferon production was assayed.

C^{14} -leucine was unaffected by the addition of the heat-inactivated virus.

Therefore, with all three experimental conditions, the amount of interferon produced was decreased in the group of cells with, if anything, the higher rate of protein synthesis.

Timing of interferon production stimulated by inactivated virus. In Fig. 1 it may be noted that no interferon was detected in culture fluids before 7 hr after infection with virus. It was also important to place interferon production as stimulated by inactivated virus in its temporal relationship to the increased levels of protein synthesis noted in the systems studied.

In the experiment summarized in Table 2, CEF were stimulated to make interferon by heat-inactivated Chikungunya virus. After 4 hr, fluids were assayed for interferon, and then 1 μ g/ml of actinomycin D was added for 1 hr and the cells were washed. After 17 additional hr of incubation, the tissue fluids were collected and assayed for interferon. Under these conditions, CEF produced interferon by the end of the 21-hr period; no interferon was found in the fluids collected 4 hr after the addition of virus; moreover, when actinomycin D was added even as late as 4 hr after infection, interferon production was blocked as completely as in cells to which actinomycin D had been added before infection.

These results suggested that the bulk of the interferon produced in the systems studied was made *after* the time when the greatest differences in protein synthesis were noted between pretreated and control cells.

TABLE 2. Induction of interferon by heat-inactivated Chikungunya virus

Treatment*	Incubation period	Interferon titer
	<i>hr</i>	<i>units</i>
Control.....	4	<4
Control.....	21	30
Actinomycin D, before infection.....	21	<4
Actinomycin D, 4 hr after infection.....	21	<4

* Chick embryo fibroblasts (5×10^6) were infected with 1 ml of a pool of heat-inactivated (36 C, 23 hr) Chikungunya virus. After 1 hr for adsorption, virus was washed off and replaced with medium. Actinomycin D (1 μ g/ml) was added to the indicated plates 2 hr before or 4 hr after virus infection. After incubation, interferon was titrated by the vaccinia plaque reduction method.

DISCUSSION

These observations indicate that the treatment of CEF with FPA or incubation at 4 C before infection with live or inactivated virus decreased the ability of the cells to produce interferon. In the case of live virus infection, this was usually correlated with an increase in the yield of virus. Paradoxically, cells which had received these treatments before infection appeared to incorporate C^{14} -leucine into protein at a more rapid rate than did untreated controls. In a system in which protein synthesis could be directly controlled by the level of serum in the medium, interferon production again appeared to vary inversely with amino acid incorporation; however, in all three experimental situations, the production of interferon seemed to lag several hours behind the maximal differences noted in protein synthesis. The results in general appeared to indicate that stimulation of protein synthesis did not necessarily give rise to optimal conditions for interferon production, since the amount of interferon produced was not directly related to the level of general protein synthesis under conditions where the cell number was constant.

The rise in protein synthesis might have nothing at all to do with interferon production but be instead an important negative finding, in that it is an instance where the production of at least one specific protein was inhibited under conditions where general protein synthesis was not. If the apparent rise in protein synthesis was related to the inhibition of interferon production, these observations are difficult to interpret. Two explanations which do have the virtue of being consistent with all of the data will be discussed; however, it is understood that other reasonable explanations are not unlikely. In the first, interferon production is assumed to be under the control of a stable endogenous repressor, since interferon does indeed appear to be an induced protein as it is not normally present in cells, tissue fluids, etc.; it is formed after exposure of cells to active or inactivated viruses or, possibly, to non-viral substances; and, its formation requires synthesis of RNA and new protein under the control of the cell genome.

The increase in protein synthesis, which for the most part preceded interferon production, might lead to accumulation of repressor within cells, since the number of cells in the confluent monolayers employed was constant and the proposed repressor is assumed to be stable. The accumulation of repressor would then make it more difficult to induce interferon production, but this could be overcome by increasing the amount of

inducer (i.e., virus). This might explain the observation that raising the multiplicity of virus by 200 times negated the effect of FPA pretreatment (Table 1, line B). Several other examples of preferential effects on specific proteins may be found (6, 15, 17). Interestingly enough, many of these involve preferential inhibition of the synthesis of induced enzymes.

On the other hand, the increase in protein synthesis may be only indirectly related to interferon production and represent a preferential synthesis of certain vital cell constituents depleted by cold, FPA, or low serum treatment. This would be analogous to the preferential synthesis of ribosomal protein occurring during recovery of bacterial cells from pretreatments inhibiting protein synthesis with respect to RNA synthesis (2, 14; Nakada, J. Mol. Biol., *in press*). The observed inhibition of interferon production might be due to a priority system on the basis of cell requirements, and the actual working mechanism of this system could involve an endogenous repressor as postulated in the previously discussed model system.

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LITERATURE CITED

1. AMOS, H., B. ASKONAS, AND R. SOEIRO. 1964. Evidence for messenger activity of alien ribonucleic acid in chick cells. *Natl. Cancer Inst. Monograph* **13**:155-165.
2. ARONSON, A. I., AND S. SPIEGELMAN. 1961. On the nature of the ribonucleic acid synthesized in the presence of chloramphenicol. *Biochim. Biophys. Acta* **53**:84-95.
3. BALTIMORE, D., AND R. M. FRANKLIN. 1963. Effects of puromycin and p-fluorophenylalanine on mengovirus ribonucleic acid and protein synthesis. *Biochim. Biophys. Acta* **76**:431-441.
4. FREIDMAN, R. M. 1964. Role of interferon in viral interference. *Nature* **201**:848-849.
5. FRIEDMAN, R. M., AND J. A. SONNABEND. 1964. Effect of p-fluorophenylalanine on interferon action. *Nature* **203**:366-367.
6. GALLANT, J., AND R. STAPLETON. 1964. Depression of alkaline phosphatase synthesis by chloramphenicol and canavanine inhibition. *J. Mol. Biol.* **8**:442-451.
7. GIFFORD, G. E., AND E. HELLER. 1963. Effect of actinomycin D on interferon production by active and inactive Chikungunya virus in chick cells. *Nature* **200**:50-51.
8. HELLER, E. 1963. Enhancement of Chikungunya virus replication and inhibition of interferon production by actinomycin D. *Virology* **21**:652-656.
9. ISAACS, A. 1963. Interferon. *Advan. Virus. Res.* **10**:1-38.
10. ISAACS, A., R. A. COX, AND Z. ROTEM. 1963. Foreign nucleic acids as the stimulus to make interferon. *Lancet* **2**:113-116.
11. ISAACS, A., AND J. LINDENMANN. 1957. Virus interference. I. The interferon. *Proc. Roy. Soc. (London) Ser. B* **174**:258-267.
12. KLEINSCHMIDT, W. J., J. C. CLINE, AND E. B. MURPHY. 1964. Interferon production induced by statolon. *Proc. Natl. Acad. Sci. U.S.* **52**:741-744.
13. LINDENMANN, J., AND G. E. GIFFORD. 1963. Studies on vaccinia virus plaque formation and its inhibition by interferon. III. A simplified plaque inhibition assay for interferon. *Virology* **19**:302-309.
14. SELLS, B. H. 1964. RNA synthesis and ribosome production in puromycin-treated cells. *Biochim. Biophys. Acta* **80**:230-241.
15. SPOTTS, C. R. 1962. Physiological and biochemical studies on streptomycin dependence in *Escherichia coli*. *J. Gen. Microbiol.* **28**:347-365.
16. STINEBRING, W. R., AND J. S. YOUNGNER. 1964. Patterns of interferon appearance in mice injected with bacteria or bacterial endotoxin. *Nature* **204**:712.
17. SYPHERD, P. S., N. STRAUSS, AND H. P. TREFFERS. 1962. The preferential inhibition by chloramphenicol of induced enzyme synthesis. *Biochem. Biophys. Res. Commun.* **1**:477-481.
18. TAYLOR, J. 1965. Studies on the mechanism of action of interferon. I. Interferon action and RNA synthesis in chick embryo fibroblasts infected with Semliki Forest virus. *Virology* **25**:340-349.
19. WHEELOCK, E. F. 1965. Interferon-like virus inhibitor induced in human leukocytes by phytohemagglutinin. *Science* **149**:310-311.
20. YOUNGNER, J. S., AND W. R. STINEBRING. 1964. Interferon production in chickens injected with *Brucella abortus*. *Science* **144**:1022-1023.