further after 8, 30 and 60 days of altitude exposure.

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A Sensitive Method for Interferon Assay.* (28735)

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In cells inaccessible to some intact viruses, virus synthesis can be induced by inoculation of RNA extracted from the virus(1,2). Since the infection with viral RNA in such cells results in only one cycle of virus multiplication and since the virus synthesis can be inhibited by interferon(3,4,5) such a cell-virus system might be used for a sensitive assay of interferon. It has also been demonstrated(5) that interferon induces a high antiviral activity against poliovirus RNA in chick cells.

This report describes the effect of interferon on poliovirus RNA infection in calf kidney cells. The dose-response relationship between interferon and the inhibition of virus reproduction is analyzed and the sensitivity of the assay thus obtained is compared with other methods for titration of interferon.

Materials and methods. Viruses. Two plaque purified strains of poliovirus type 1 have been used, the virulent strain E 206 and the attenuated strain Lsc 2 ab. The stocks of these viruses were made in KB cells. Stock suspensions of strain 205 of Newcastle disease virus (NDV), the Sendai strain of parainfluenza virus type 1 and a strain of foot-and-mouth disease virus (FMDV) type A5 were prepared in calf kidney cells. Poliovirus and NDV were stored at +4°, the other viruses at −60°.

Cell cultures and virus assays. Cultures of trypsin-dispersed calf kidney cells were prepared as previously described(6). Hanks' salt solution with 0.5% lactalbumin hydrolysate (Hanks' + Lah) and 10% calf serum were used for the outgrowth and Eagle's minimum essential medium (MEM)(7) for maintenance of the cells. KB and Hela cells were prepared as described elsewhere(8).

Plaque assay of poliovirus and NDV was performed in Hela cells grown in plastic petri dishes. The overlay contained MEM, 10% horse serum and 0.95% agar (Special Agar Noble, Difco). FMDV was assayed in calf

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kidney cells with an overlay containing Earle's salt solution, 0.05% lactalbumin hydrolysate, 2% calf serum and 0.95% agar.

Hemagglutinin (HA) titrations were performed with guinea pig erythrocytes in tubes and the titer was expressed as the highest initial dilution of the sample giving partial agglutination(10).

Extraction and assay of viral RNA. KB cells were inoculated with poliovirus at a multiplicity of about 10 and incubated for 18 hours at 37° with MEM. The cell fraction was lysed by addition of 8 M urea and diluted ten-fold in 0.1 M phosphate buffer pH 7.2. The lysed cells were centrifuged at 2,600 *g* for 10 minutes. The supernatant, containing about 10^9 PFU/ml poliovirus, was extracted once with phenol saturated with 0.1 M phosphate pH 7.2 and subsequently 5 times with ether to remove excess phenol. The ether was finally removed with nitrogen. The RNA preparations were stored at –60° and were used within 3 weeks.

The infectivity of the RNA preparations was assayed by the plaque technique and the infectious RNA was adsorbed to the cells in 2 M $MgSO_4$. Between 0.1-0.3% of the infectivity of the intact virus was recovered in the viral RNA.

Preparation of interferon. NDV was inoculated into calf kidney cultures at a multiplicity of about 0.01. The culture fluids were harvested after incubation for 4 days at 37° and saturated to 75% with ammonium sulphate. The precipitate obtained after 18-24 hours at 4° was sedimented by centrifugation at 1,500 *g* for 30 minutes. The pellet was resuspended in Hanks' + Lah and dialyzed against Earle's salt solution. Viral activity was removed by 2 centrifugations at 85,000 *g* for 2 hours. The interferon preparations were stored at –60° and remaining infectivity was neutralized prior to use by addition of NDV antiserum.

FMDV interferon was prepared in calf kidney cultures as described previously(9) with inactivation of the infectious virus by dialysis at pH 2.5.

Assay of interferon. Primary cultures of calf kidney cells in plastic petri dishes were inoculated with different dilutions of interferon in MEM. When NDV interferon was tested the medium contained 20 hemagglutination-inhibition units of NDV antiserum in order to neutralize remaining viral activity. The plates were incubated for 18-24 hours at 37°, the medium was then carefully removed and the cultures inoculated with about 1000 PFU of poliovirus RNA in 0.1 ml 2 M $MgSO_4$. After adsorption of viral RNA for 10 minutes at room temperature, the plates were washed twice with Hanks' + Lah and supplied with fresh medium. The yield of poliovirus was determined by plaque assay of the medium after a fixed period of incubation at 37°. Only the medium was assayed since no difference in titer was obtained when the cell and the fluid phases were assayed separately.

For comparison, the interferon preparations were also tested by methods based upon the inhibition of plaque formation and the reduction of virus yield after infection with active virus. In these tests which have previously been described(9,10), the calf kidney cultures were also treated with interferon for 18-24 hours prior to the challenge infection. The challenge viruses were FMDV and Sendai virus respectively.

Results. Effect of interferon on viral RNA. In an assay based upon inhibition of virus reproduction in cells treated with interferon and infected with viral RNA, it must be excluded that interferon inactivates the infectivity of the RNA preparations. Poliovirus RNA from the strain E 206 was diluted in 2 M $MgSO_4$ and mixed with an equal amount of undiluted NDV interferon. The mixture was incubated for 10 minutes at room temperature and assayed for infectivity. The influence of ammonium sulphate on viral RNA was also studied since the interferon preparations were concentrated with this salt. Viral RNA was diluted in 2 M $MgSO_4$ containing different concentrations of $(NH_4)_2SO_4$ and assayed by the plaque technique.

Table I shows that the infectivity of the RNA preparation was not influenced by incubation with the interferon preparation or 0.01 M ammonium sulphate. Presence of 0.1

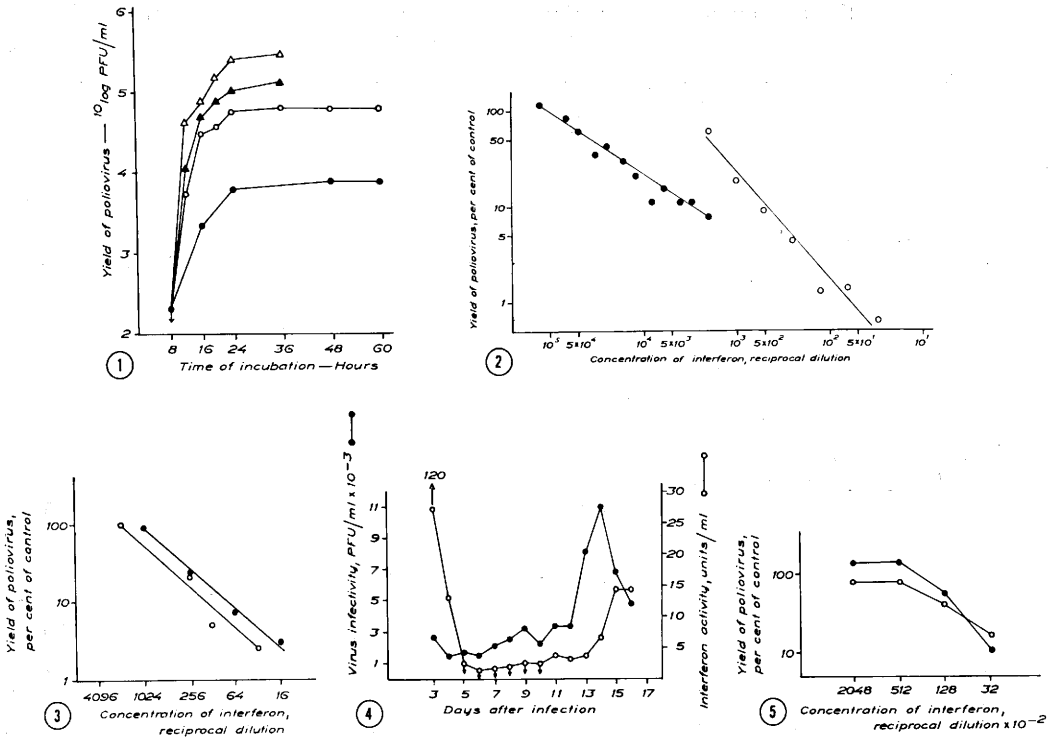

FIG. 1. Rate of production of poliovirus infectivity in calf kidney cells at different concentrations of NDV interferon. △——△, No interferon; ▲——▲, interferon diluted 1/32,000; ○——○, interferon diluted 1/4,000; ●——●, interferon diluted 1/500.

FIG. 2. Dose-response relationship between poliovirus yield and concentration of NDV interferon in calf kidney cells. ●——●, NDV interferon preparation I with high interferon activity; ○——○, NDV interferon preparation II with low interferon activity.

FIG. 3. Dose-response relationship between poliovirus yield and concentration of FMDV interferon in calf kidney cells. ●——●, FMDV interferon preparation I; ○——○, FMDV interferon preparation II.

FIG. 4. Production of interferon and infectious virus in cells persistently infected with NDV.

FIG. 5. Dose-response relationship between poliovirus yield and concentration of NDV interferon with virulent and attenuated poliovirus RNA as challenge. ●——●, Virulent viral RNA, strain E 206; ○——○, attenuated viral RNA, strain LSc 2ab.

M $(NH_4)_2SO_4$ in the inoculum of the viral RNA reduced the infectivity 47%. The concentrations of ammonium sulphate were lower, however, than 0.01 M in the interferon preparations after dialysis.

Rate of virus production at different concentration of interferon. This was followed in plate cultures of calf kidney cells treated with different dilutions of NDV interferon and challenged with poliovirus RNA from the strain E 206 as described in Methods. Samples of the culture fluids were removed after different periods of incubation and assayed for infectivity.

As shown in Fig. 1 the yield of poliovirus was suppressed at higher concentrations of interferon. Maximum titers were attained in all cultures 24 to 36 hours after the infection with viral RNA. The rate of virus synthesis expressed in percentage yield per unit time was not significantly different at various concentrations of interferon.

TABLE I. Effect of Interferon and Ammonium Sulphate on Infectivity of Poliovirus RNA.

Treatment	Infectivity in PFU/ml		
	Controls	Treated	%
Interferon	2.3×10^4	2.1×10^4	9
.1 M $(NH_4)_2SO_4$	1.4×10^5	7.5×10^4	47
.01 M $(NH_4)_2SO_4$	1.4×10^5	1.5×10^5	0

TABLE II. Correlation and Regression Coefficients for Dose-Response Relationship Between Logarithm of Concentration of Interferon and Logarithm of Virus Yield.

NDV interferon preparation	Correlation coeff	Regression coefficient		Standard error of estimate	
		y on x	x on y	S_y	S_x
I	−.88	−.58	−1.34	.18	.27
II	−.84	−.92	− .76	.38	.35

y = log virus yield. x = log interferon concentration.

Dose-response relationship. The relationship between dose of interferon and yield of poliovirus in calf kidney cultures infected with poliovirus RNA was studied according to the technique described in Methods. Two preparations of NDV interferon with different activity were tested. Each dilution of interferon was inoculated into 3 separate plate cultures and the culture fluids were assayed for infectivity after incubation with RNA from the strain E 206 for 18 hours. Virus assays were performed by the plaque technique with one plate for each 10-fold dilution.

The results are recorded in Fig. 2 in which the reciprocal dilution of interferon is plotted against the reduction in virus yield. Each point represents the mean value of the yield from 3 plates. The results suggest a linear relationship between the logarithm of the interferon concentration and the logarithm of virus yield in per cent of control. The correlation coefficients were calculated to be −0.88 and −0.84, respectively, according to the method of least square. The regression coefficients and the standard error of estimate are recorded in Table II.

Different regression coefficients were obtained for the two interferon preparations. The difference, however, is not significant ($0.1 > P > 0.05$).

Evidence for a linear relationship between the log concentration of interferon and the log reduction of virus yield was also obtained with FMDV interferon (Fig. 3). The regression coefficient for the two preparations tested was about 1.0.

Sensitivity of assay. The sensitivity of the described assay system was compared with other methods used in this laboratory for assay of interferon activity(9,10). The different interferon preparations were tested for their capacity to suppress the yield of Sendai virus and plaque formation by FMDV in calf kidney cultures. The interferon titer was expressed as the highest reciprocal dilution of interferon which reduced the yield of Sendai virus hemagglutinin by 75% and the number of FMDV plaques by 50%. In the RNA-interferon test the titer was expressed as the reciprocal dilution of interferon which induced a 50% reduction of the yield of poliovirus. The results are recorded in Table III which shows that the RNA-interferon method is about 100 times more sensitive than the others.

Due to the insensitivity of the interferon assays it has been difficult to demonstrate interferon in persistently infected cells in which virus synthesis is strongly inhibited (11,12,13). It was therefore of interest to follow the production of interferon with the RNA-interferon test in calf kidney cultures persistently infected with NDV. A persistent infection was established by inoculation of plate cultures with a multiplicity of about 1 as reported previously(14). The medium was changed daily and assayed for infectivity and interferon activity as described in Methods.

TABLE III. Comparison of Different Methods for Assay of Interferon.

Preparation	Reciprocal dilution of interferon		
	75% reduction in yield of Sendai virus	50% plaque reduction of FMDV	50% reduction in yield of poliovirus
NDV interferon preparation I	512	N.D.	36000
" " " II	32	16	2000
FMDV " " II	4	<4	1000

N.D. = not done.

The interferon titer, *i.e.*, the dilution which caused a 50% reduction of the virus yield, was calculated from the reduction of the poliovirus yield in one dilution and from the regression coefficient established for 2 of the samples.

As seen in Fig. 4 the interferon activity appears in cycles. Virus synthesis is also cyclic and the synthesis of interferon seems to occur simultaneously with, or somewhat later than, the production of virus.

Since it has been reported that avirulent strains of some viruses are more sensitive to interferon than virulent strains(15,16) it was investigated whether it was possible to get a more sensitive assay with an avirulent strain of poliovirus. NDV interferon was assayed with viral RNA from the attenuated strain Lsc 2 ab and the virulent strain E 206 as described above. Fig. 5 shows that in the system used no clear difference in sensitivity could be observed between the two strains of poliovirus.

Discussion. To develop a sensitive method for assay of interferon the relation between concentration of interferon and virus yield has been studied on calf kidney cultures infected with poliovirus RNA. Preincubation of the cells with interferon suppressed virus production but no significant delay of the virus synthesis was observed. Maximum virus yields were obtained after 24 to 36 hours at 37°, a period of incubation which appears suitable when interferon is assayed in this system.

When virus yield was assayed after a fixed period of incubation a linear relationship was found between the logarithm of the dose of interferon and the logarithm of the virus yield. A similar linear relationship between log virus yield and log dose of interferon was obtained in L cells with intact Western equine encephalitis virus(17). In other systems, however, evidence for single-hit kinetics(18,19) has been presented. Since impure preparations of interferon has been used in these studies it is uncertain whether interferon acts by single-hit or a more complex mechanism.

The linear relationship between the logarithm of the concentration of interferon and that of the virus yield in the RNA-interferon test, however, is useful for assay purposes since the interferon titer can be calculated simply from the reduction in virus yield in one dilution when the regression is known. The interferon titer can then preferably be expressed as the reciprocal dilution of the sample which reduces the virus yield by 50%. This method for estimation of interferon activity is complicated by the fact that it is uncertain whether different preparations of interferon give the same regression coefficient. For an adequate titration, therefore, it is necessary to determine the regression for each sample although for practical purposes it might be justified to determine the regression for each experimental system. It is also advisable to calculate the interferon titer from dilutions giving 50 to 95% reduction in virus yield since within this range moderate deflections in regression give only minor errors of the interferon titer.

The RNA-interferon test was found to be about 100 times more sensitive than other methods investigated for assay of interferon (9,10). The sensitivity of the assay system is also illustrated by the detection of interferon in calf kidney cultures persistently infected with NDV in which the virus yield was suppressed to less than 99.9% of the normal yield in these cells(14).

Summary. A method for assay of interferon activity based on the inhibition of virus yield in calf kidney cultures infected with poliovirus RNA is described. Evidence has been obtained for a linear relationship between the logarithm of the dilution of interferon and the logarithm of the virus yield suggesting a complex nature of the action of interferon. The RNA-interferon test was about 100 times more sensitive than other methods tested. This method also detected interferon in calf kidney cultures persistently infected with NDV.

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Carcinogenic Response of the Syrian Golden Hamster Treated at Birth With 7,12-Dimethylbenz(a)anthracene.* (28736)

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High incidences of malignant lymphomas and lung adenomas and a few other tumors have been obtained in mice treated at birth with chemical carcinogens(1,2,3,4,5,6,7,8,9). It becomes apparent from some of these studies that 7,12-dimethylbenz(a)anthracene (DMBA) is particularly powerful in evoking malignant lymphomas in several strains of mice treated at birth.

The effects of DMBA, administered at birth in different species, are being studied in this laboratory. In Lewis rats, it gave rise to subcutaneous sarcomas at the site of injection(10).

The present paper describes the action of DMBA given subcutaneously to newborn Syrian golden hamsters. In adult Syrian golden hamsters subcutaneous administration of DMBA induced sarcomas(11), while cutaneous application resulted in the development of dermal melanocytomas(12).

Materials and methods. Syrian golden hamsters from a colony originally obtained from Abrams Small Stock Breeders, Chicago, Ill., and bred randomly in this laboratory since 1959, were used. Each litter was housed with its mother until it was weaned, and was then separated according to sex. The hamsters were housed in plastic cages on sterilized granular cellulose bedding in groups not exceeding 6 to a cage, fed Rockland diet in pellets and tap water *ad libitum*.

The carcinogen used was 7,12-dimethylbenz(a)anthracene (DMBA),† purified by chromatography on magnesia/Celite. It was dissolved in tri-n-caprylin (trioctanoin),† purified prior to use by vacuum distillation. The newborn animals were given a single subcutaneous injection in the interscapular region with a tuberculin syringe using a 30-gauge needle. Concentrations of DMBA were such that the desired dose was contained in 0.05 ml of tri-n-caprylin. Each DMBA treated animal was checked for a few seconds under a U.V. lamp to assure that no free DMBA had leaked on the skin or was left in the needle track. Those animals which showed fluorescent material on the skin were discarded. The injections were made less than 4 hours after birth of the ani-

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† Eastman Organic Chemicals, N. Y.