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Growth of Rubella Virus in BHK21 Cells. II. Enhancing Effect of DEAE-Dextran, Semicarbazide and Low Doses of Metabolic Inhibitors.* (32284)

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The enhancement of virus growth in animal cell cultures by changes in environmental conditions, by various chemical substances, by physical treatment and by "helper" viruses, has been actively studied recently. Such enhancement phenomena, which have obvious bearing on the control mechanisms of viral multiplication, have also been of practical significance in the laboratory investigation of several viruses. The foregoing report(1) described conditions for the study of rubella virus (RV) in tissue cultures of the WI-2 and 13S clones of baby hamster kidney BHK21 cells. In this paper we show that RV growth and study may be further improved, even in these highly susceptible cell systems, by addition of diethylaminoethyl-dextran (DEAE-dextran) and semicarbazide (SCZ) and to some degree also by small doses of Actinomycin D (ACTD), 5-Fluoro-2'-Deoxyuridine (FUdR) and UV pretreatment of host cells. Experimentation with DEAE-dextran was prompted by the finding that it enhances the infectivity of poliovirus and poliovirus RNA(2,3), while studies of SCZ were stimulated by the work of Grossberg and Lwoff(4,5) on the exaltation of poliovirus replication.

Materials and methods. Tissue culture, media, virus stocks and assays were described in the previous paper(1). Abbreviations: CMC = carboxymethylcellulose, CPE = cytopathic effect, M = multiplicity of virus per cell (PFU/cell) in inoculum, MEM = Eagle's minimum essential medium, NDV = New Castle disease virus, PBS = Dulbecco's phosphate buffered saline, PFU = plaque forming unit(s).

Chemicals. DEAE-dextran: (diethylaminoethyl-dextran, Pharmacia), prepared from dextran with M.W. = 2×10^6. A stock

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TABLE I. Effect of DEAE-Dextran in the Inoculum on Plaquing of Rubella Virus on BHK21/WI-2 Cells.

DEAE-dextran (μg/ml)	No. of plaques*
0	12
10	13
25	38
50	34
100	28
250	20
500	22†

* Average plaque number per cell culture. Test performed under standard plaquing conditions(1) except that the plates were washed 3 times with PBS after the adsorption period of 90 min.

† Slight visual evidence of cell toxicity.

solution containing 10 mg/ml was made in PBS and was sterilized by autoclaving. *Semicarbazide:* A one-M stock solution of SCZ (Semicarbazide (NH_2-NH-CO-NH_2) hydrochloride, Baker) was prepared in distilled water, neutralized with NaOH and sterilized by passing through a Seitz filter. *Actinomycin D:* (Merck, Sharp and Dohme), a stock solution of ACTD containing 100 μg/ml was prepared in PBS and stored at 20°C in the dark. Stock solutions of *FUdR (5-Fluoro-2'-Deoxyuridine,* Hoffmann-LaRoche), *Cytosine Arabinoside* (Sigma), *Hydrocortisone* acetate (Sigma), *Prednisolone* (Sigma), *Heparin,* sodium salt (Sigma) and *Colchicine* (Ciba) were prepared in PBS and stored at +4°C.

Pretreatment of BHK21/WI-2 cells with ultraviolet irradiation. The monolayer cultures from which the medium had been removed were exposed to ultraviolet light delivered by a Westinghouse Sterilamp® G 37T6L emitting more than 84% of its energy at 2536-2652 Å and less than 0.2% at 2809-2894 Å. The distance from the lamp was 23 cm.

Results. DEAE-dextran. When DEAE-dextran was added to a virus inoculum containing a countable number of PFU, there was an increase of 2- to 3-fold in the number of plaques which appeared 6 days after infection (Table I). The concentration of DEAE-dextran giving the maximum enhancement of plaque number lay between 25 and 100 μg/ml. The presence of DEAE-dextran in concentrations up to 250 μg/ml during the adsorptive phase was not detectably injurious to the cells under the conditions outlined in Table I.

To document further the effect of DEAE-dextran on the early RV-cell interaction, experiments were performed in which DEAE-dextran was present either during or after adsorption of a high multiplicity of virus. The results are given in Table II. The presence of DEAE-dextran during adsorption increased virus yields by 0.4 to 1.3 $\log_{10}$ PFU, depending on the time of sampling. Addition of the same compound 2 hours after virus inoculation, however, increased virus yield only during the first 24 hours of the growth cycle, and then less profoundly. Later in the growth cycle, no difference was seen between DEAE-dextran treated and untreated cultures.

With lower multiplicities of infection, DEAE-dextran produced a more pronounced increase in virus yield when added post-

TABLE II. Effect of Presence of DEAE-Dextran During and After Adsorption on RV Yields.

	Virus yield in $\log_{10}$ PFU/ml — Hours post-inoculation									
	14		18		24		36		45	
Concentration of DEAE-D (μg/ml)	S	C	S	C	S	C	S	C	S	C
None	3.5	3.5	5.0	4.8	6.3	6.2	6.7	5.9	6.8	6.9
10 During adsorp.	4.1	3.7	5.9	5.4	6.7	6.1	6.9	5.9	7.2	7.0
25 " "	4.2	4.7	6.1	5.8	6.9	6.3	7.1	7.0	7.2	7.3
50 " "	4.2	4.5	6.3	5.8	6.9	6.4	7.0	6.9	7.1	7.3
10 Post adsorp.	3.4	3.7	5.1	5.1	5.8	6.1	6.7	5.9	6.8	6.9
25 " "	3.8	3.9	5.2	5.4	6.5	6.1	6.5	6.0	6.7	6.9
50 " "	3.8	4.0	6.1	5.3	6.6	6.1	6.9	6.1	6.6	7.0

S = supernatant. C = cell-associated. Determined by sonication for 3 min at 1 ampere in a Raytheon Magnetostrictive Sonic Oscillator Model DF 101.

TABLE III. Effect of DEAE-Dextran and Other Compounds on the Virus Replication and Development of CPE in Rubella Virus-Infected BHK21/WI-2 Cell Cultures.

Multiplicity (M) of infection		Virus yield (V), degree of CPE							
		24 hr		48 hr		72 hr		96 hr	
Compound‖	Concentration	V*	CPE†	V	CPE	V	CPE	V	CPE
M = 2 PFU/cell									
Control	——	5.6	—	7.3	±	6.9	+		
DEAE-dextran	10 μg/ml	6.4	—	8.0	++	7.3	+++		
	5 μg/ml	6.3	—	7.6	++	7.3	+++		
	2.5 μg/ml	6.5	—	7.4	+	7.0	+++		
	1 μg/ml	6.1	—	6.9	±	7.1	++		
Semicarbazide	2.5 mM	5.5	—	6.8	+	7.3	++		
Prednisolone	2.5 μg/ml	5.7	—	7.3	—	7.3	±		
M = 0.2 PFU/cell									
Control	——	5.3	—	7.1	—				
DEAE-dextran	10 μg/ml	5.9	—	7.2	—				
Heparin	300 μg/ml	5.2	—	7.1	—				
Actinomycin D‡	.3 μg/ml	5.5	—	6.2	Toxic				
	.03 μg/ml	6.0	—	7.1	—t§				
	.003 μg/ml	5.5	—	7.4	—				
FUdR	.1 μg/ml	5.6	—	6.3	—t				
	.01 μg/ml	5.4	—	7.3	—				
Colchicine	.5 μg/ml	5.4	—	7.1	—t				
Hydrocortisone	100 μg/ml	5.4	—	7.1	—				
M = 0.02 PFU/cell									
Control	——			5.3	—			7.1	+
DEAE-dextran	10 μg/ml			6.2	—			7.2	++
	5 μg/ml			6.2	—			7.5	++
	2.5 μg/ml			5.8	—			7.6	++
	1 μg/ml			5.8	—			7.6	++
Semicarbazide	2.5 mM			5.2	—			7.0	++
Prednisolone	2.5 μg/ml			5.3	—			7.2	+

* Total virus yield (extra- and intracellular, PFU/ml, $\log_{10}$) in cultures. Obtained by harvesting cells with medium and subjecting them to 3 freeze-thaw cycles.
† Degree of rubella virus CPE in arbitrary units.
‡ Actinomycin D-treated plates were incubated in the dark.
§ Slightly toxic.
‖ Added 2 hr post-infection.

adsorption. As summarized in Table III, virus yields from cultures maintained in the presence of DEAE-dextran were higher than in the absence of this polycationic compound. The enhancement by DEAE-dextran of the evolution of CPE was even more striking than the actual increase in viral yields. The toxicity level of DEAE-dextran in liquid medium showed some variation from one experiment to another, ranging between 25 and 100 μg/ml.

The addition of 300-3000 μg/ml of DEAE-dextran to the CMC overlays appeared to improve the plating efficiency by increasing both the number and size of RV plaques. However, these concentrations had toxic effects on the monolayers and produced cloudiness in the overlays, apparently caused by a reaction between the DEAE- and carboxy-groups of the oppositely charged polymers. In 0.2% agarose overlay medium, as little as 10 to 30 μg/ml of DEAE-dextran enhanced the plaquing efficiency by 2-fold; the higher concentration, however, was also toxic.

In view of the polycationic configuration of DEAE-dextran it was of interest to test the effect of an oppositely charged polymer. The polyanion heparin, in a concentration known to inhibit several viruses(8), had no effect on RV replication or the progress of viral CPE (Table III).

Semicarbazide. This compound proved useful in the assay of RV. Incorporation of

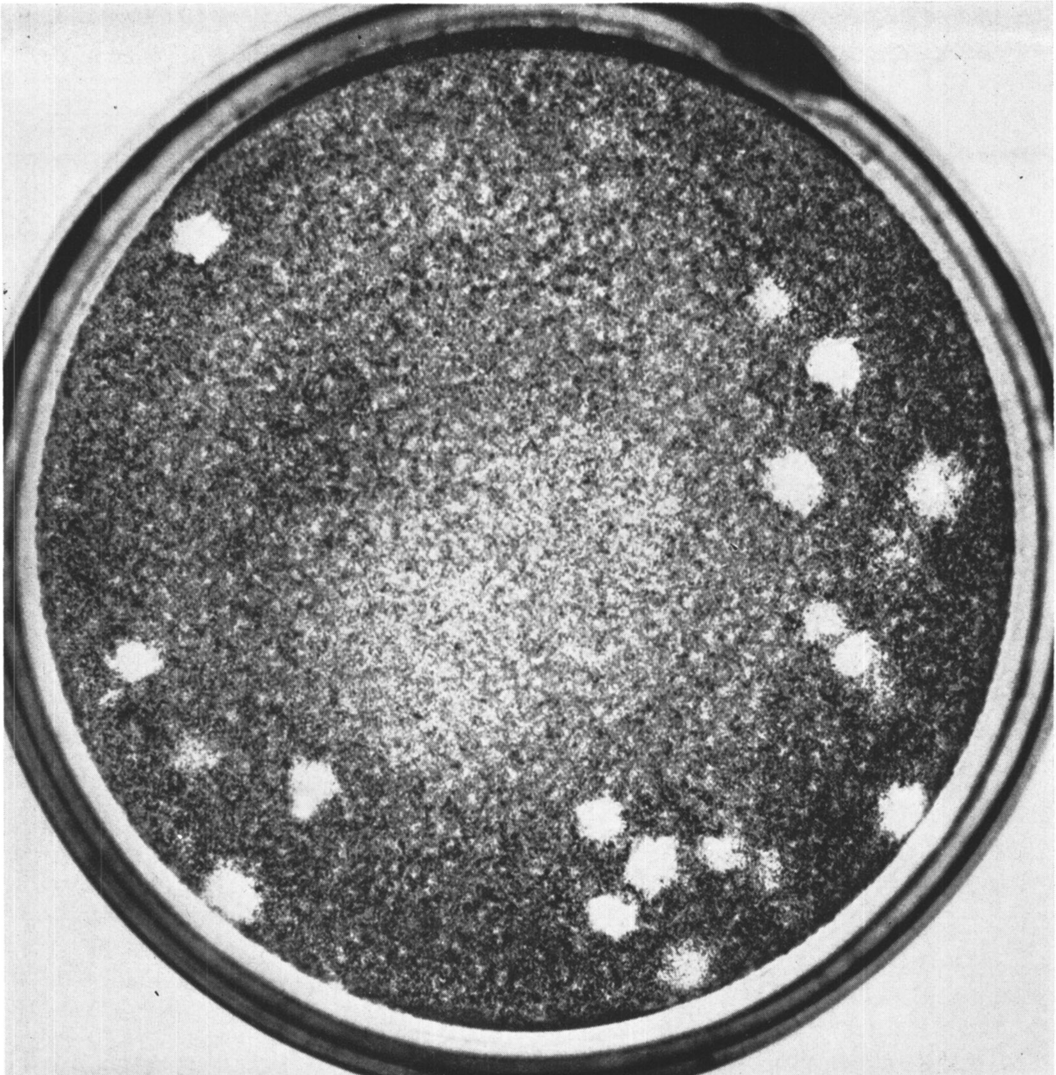

Fig. 1. Plaque of RV strain RA27/3 in BHK21/WI-2 cells. The overlay contains Eagle's medium, 2% fetal calf serum, 0.75% carboxyethylcellulose, and 2mM semicarbazide. Original diameter 60 mm.

SCZ in a concentration of 2.5 mM into the semifluid overlay medium containing CMC markedly improved the quality of the plaques (Fig. 1) and increased the plaque count by about 2-fold. Plaques of several different RV strains, RA27/3, H600, and West Point (1), were all clearer and more numerous when developing in the presence of SCZ. With the RA27/3 strain, the increase in plaque number varied between 1.5- to 5-fold in different tests, depending on the variable maintenance of the non-SCZ-treated cell cultures. Higher concentrations of SCZ (5-10 mM) resulted in a decreased plating efficiency in most experiments, and 1 mM was always less effective than the standard 2.5 mM concentration. When present only during adsorption of virus, SCZ had no enhancing effects on plaque development.

In liquid maintenance medium, SCZ at 2.5 mM did not produce any significant alterations in yields of RV in BHK21/WI-2 cell cultures (Table III).

The enhancing effects of SCZ and DEAE-

TABLE IV. Effect of DEAE-Dextran and Semicarbazide Titration of Rubella Virus by Cytopathic Effect in BHK21/WI-2 Cell Cultures.

		Titers observed (log_{10} TCD_{50}/ml)*									
		Days									
Compound	Concentration	4	5	6	7	8	9	10	11	12	14
Control	—	3.5	5.0	5.5	6.3	6.8	N.G.†				
DEAE-dextran	10 μg/ml	4.5	6.0	7.0	7.5	7.5	7.5	N.G.			
Semicarbazide	1 mM	3.5	5.5	5.8	6.5	7.0	7.0	7.5	N.G.		
	2 mM	3.5	5.5	6.0	7.5	7.8	7.8	7.8	7.8	7.8	N.G.
DEAE-dextran Semicarbazide	10 μg/ml 2 mM	4.5	6.0	7.0	8.0	8.5	8.5	8.5	8.5	8.5	N.G.

* Three replicate plate cultures per virus dilution.
† N.G., control cells degenerated.

dextran on the efficiency of RV plaquing suggested that they would be useful in RV titration by CPE. Table IV shows that, in fact, a combination of the two, 2 mM SCZ and 10 μg/ml of DEAE-dextran, gave the best results. Not only was the endpoint titer about 1.5 logs higher than in the control, but also the maintenance of adequate monolayers, and hence the clarity of the cytopathic effect, was substantially improved. With DEAE-dextran or SCZ alone, the improvement in titer and cell maintenance was not as pronounced.

Cellular inhibitors. Previous studies from this laboratory have demonstrated the lack of effect on virus synthesis of addition of 2.5 μg/ml ACTD to rubella infected RK_{13} and green monkey kidney cell cultures(9). BHK21/WI-2 and 13S cells were found to be more sensitive to ACTD, and 0.3 μg/ml markedly depressed host-cell RNA synthesis. When added after viral adsorption, 0.3 μg/ml of ACTD gave only slight or no depression of RV yields in BHK21 cell cultures harvested at 24 hours, just before signs of toxicity (Table III). On the contrary, a slight enhancing effect of the drug on virus production and on the development of viral CPE was regularly observed at lower concentrations, particularly at 0.01-0.03 μg/ml (Table III).

In several endpoint titrations of RV the presence of 0.01-0.03 μg/ml of ACTD (not tabulated) increased the titers observed. The inconvenience of incubating plates in the dark and the relatively high toxicity of the drug to BHK21/WI-2 cells made this system impractical for virus titration.

Low doses of ACTD could be used without toxicity in the plaquing overlay medium for plaques if the plates were incubated in the dark. At 0.003 μg/ml the antibiotic increased the number of plaques by 2.5-fold and the plaque size by about 50% when read on day 6 (Table V). Similarly, plaque size and number were enhanced by incorporation of FUdR at 0.003 μg/ml in the overlay (Table VI). Cytosine arabinoside in concentrations which were known to inhibit rabies virus replication in BHK21 cells(13) neither inhibited nor decreased plaque formation of rubella virus in BHK21/WI-2 cells (Table VI).

Except for effects induced by toxicity, incorporation of FUdR or colchicine into fluid media did not decrease virus yields or affect development of RV CPE in BHK21 cells (Table III).

Pretreatment of BHK21 cultures with UV irradiation for 5 to 10 seconds resulted in an increased RV yield of about 0.5 log_{10} PFU.

Exposure to 20 seconds of UV under the conditions described in *Materials and methods* had no effect on virus yield, while 60 seconds of UV produced a definite decrease.

Corticosteroids. Corticosteroids are known

TABLE V. Effect of Incorporation of Actinomycin D in Overlay Medium on Plaque Assay of Rubella Virus in BHK21/WI-2 Cells.

Actinomycin D (μg/ml)	Plaques in average on 6th day: No.	Size in mm
0	18	2–2.5
.001	24	2–2.5
.003	47	3–4
.01	Many*	Large*
.03	Toxic	Toxic

* Not readable due to irregular toxicity.

TABLE VI. Effect of Incorporation of FUdR and Other Compounds in the Overlay Medium on Plaque Assay of Rubella Virus in BHK21/WI-2 Cells.

Compound	Concentration (μg/ml)	Plaques in average on 6th day	
		No.	Size in mm
Control	—	14	2
FUdR	.003	15	2
	.01	22	2.5
	.03	29	3–3.5
	.1	21	2.5
	.3	Toxic	Toxic
Cytosine arabinoside	1	13	2
	3	11	2
	10	Toxic	Toxic
Prednisolone	1	13	1.5–2
	2.5	12	1 –1.5
	5	12	1 –1.5

to modify several virus-cell interactions(10) and more specifically to stabilize lysosomal membranes which protect the cell against damage inflicted by lysosomal enzymes(11, 12). In the RV-BHK21/WI-2 cell system 2 corticosteroid derivatives were tested: hydrocortisone and prednisolone, both of which partially mitigated the viral CPE without a concomitant drop in virus yields (Table III). In agreement with the retarding effect of corticosteroids on CPE, incorporation of prednisolone in the overlay medium resulted in a decreased plaque size (Table VI).

Discussion. This paper describes several ways to enhance the replication and improve the assay of RV in BHK21 cells which are basically highly susceptible to RV(1). Enhancement of RV replication in BHK21 cells may aid in detection of RV in clinical specimens, in preparation of high-titered virus and antigen stocks, and in the study of virus specific metabolic and macromolecular changes in RV-infected cells.

DEAE-dextran is a polycation of considerably high molecular weight (more than 2×10^6) and has been used previously in viral studies for several different purposes: (a) to neutralize the inhibitory effect of agar polysulfates in the overlay medium(14), (b) to enhance the infectivity of viral nucleic acids, and (c) to increase the infectivity of poliovirus(2,3). The findings reported herein show that DEAE-dextran augments the infectivity, replication and, to some extent, the plaque formation of RV. The clear-cut enhancing effect of DEAE-dextran on virus infectivity in the adsorption experiments and in the endpoint titrations suggests that it acts at least in part during the early virus-cell interaction. Although the exact mechanism of action of DEAE-dextran was not determined, the enhancing effect of DEAE-dextran on RV was clearly separate from the indirect enhancement of viral plaque formation through neutralization of polyanionic agar polysulfates(14).

Semicarbazide, when added during the first 3 hours of viral development, enhances replication of poliovirus(4). In single-cycle growth experiments SCZ both diminished the latent period, and increased the rate of production and yield of poliovirus. According to Lwoff(4) and Grossberg *et al*(5), SCZ acts by increasing the formation or activity of RNA polymerase and by influencing the nutritional factors which affect viral development. The mechanism of the effect of SCZ on RV is obscure, since SCZ did not markedly increase the virus yield but did increase plaque size.

Our results provide additional evidence for classifying RV, a large ether-sensitive RNA virus in Group II myxoviruses. Members of myxovirus Group I (influenza, fowl plaque) are inhibited by ACTD; however, rubella virus, like Group II myxoviruses (NDV, parainfluenza) and measles, is unaffected or enhanced by this antibiotic(19). In agreement with previous findings(20) FUdR did not inhibit the replication of RV in BHK21 cells. These data suggest that no DNA-directed mechanisms are involved in the multiplication of RV.

Furthermore, UV irradiation of host cells rapidly reduces their capacity to produce fowl plaque virus, but similar treatment does not affect the yield of NDV(21). In the present study a suitable degree of UV irradiation increased RV production.

The enhancement of myxoviruses by low concentrations of DNA inhibitors is conceivably due to interference with cellular nucleic acid synthesis so that more RNA derived from penetrated virus may replicate

(21). It is also possible that the effect of nucleic acid inhibitors is due to inhibition of interferon production. In the RV-BHK21 cell system, however, this is unlikely, as no interference appears to be induced against vesicular stomatitis and Newcastle disease viruses (22). The enhancement of RV by ACTD and FUdR might also be due in part to moderation of the overall cell metabolism in cell cultures and resultant better maintenance.

Summary. The excellent growth of rubella virus in BHK21 cells is further enhanced by a number of substances. DEAE-dextran increased the plaquing efficiency, yield of virus, and cytopathic effect of the virus, particularly when present during virus adsorption. The incorporation of semicarbazide into maintenance media improved plaquing efficiency and cytopathic effect, but not virus yield. Other substances such as actinomycin and 5 fluoro-2′deoxyuridine, when present in low dosages in the maintenance media of infected cells, increased virus yield, cytopathic effect, and plaquing efficiency.

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Growth of Rubella Virus in BHK21 Cells. III. Production of Complement-Fixing Antigens.* (32285)

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Complement-fixing (CF) antigens for rubella virus have been reported in a number of cell systems: Sever *et al*(1) reported the development of a cell-associated antigen in infected RK_{13} and African green monkey kidney cells. Soluble CF antigens were found by Schmidt and Lennette(2) in concentrated

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