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Effect of Polyions on the Infectivity of Rabies Virus in Tissue Culture: Construction of a Single-Cycle Growth Curve

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The infectivity of fixed rabies virus in a number of cell lines has been shown to be markedly enhanced by the addition of protamine or diethylaminoethyl dextran to the virus inoculum. The polycations appear to exert their influence at a very early stage (adsorption or penetration or both) of virus-cell interaction. Immune globulin blocked infection completely when added up to 5 min after exposure and almost completely when added 5 to 15 min after infection. Antibody had no effect on adsorption and penetration when added to the inoculum 30 min or more after cells were exposed to the virus. Irradiation of BHK/21 cell monolayers with ultraviolet light increased their sensitivity to rabies virus. The events occurring after synchronous infection of cells in both irradiated and nonirradiated cell monolayers were followed by means of fluorescent-antibody staining and by intracerebral titration in mice. Virus-specific fluorescent antigen first appeared between 8 and 9 hr after infection, and in irradiated cultures there was a further lag period of 3 hr before infectious virus was produced intracellularly. Virus was first detected in the medium 12 to 15 hr after infection, and maximal yield of infectious virus was observed 48 hr after exposure. In nonirradiated cultures, formation of infectious virus was delayed, and the final yield of virus was also reduced.

The availability of a number of tissue culture systems for the propagation of rabies virus has greatly facilitated studies of this agent (6, 15). The low efficiency of infection of cells and the slow development of virus within them remain, nevertheless, major obstacles.

Prior exposure of human diploid cells to lymphocytic choriomeningitis (LCM) virus has been shown to increase markedly the infectivity of rabies virus for these cells (16). Because of their low susceptibility, however, the diploid cells are not the ideal host for a study of the biological and morphological events accompanying intracellular development of rabies virus. A more useful cell line in this respect is BHK/21 (baby hamster kidney fibroblasts) (2), but the enhancing effect on rabies infectivity by LCM virus does not occur in this line (16).

A search for other substances which would enhance uptake of rabies virus in tissue culture was centered on polyions, and it was found that diethylaminoethyl (DEAE) dextran and protamine increased markedly the infectivity of the virus in human diploid cells and other cell lines examined, including BHK/21 (14*a*). In conjunction with ultraviolet (UV)-irradiation of the cells, this finding made it possible to infect 100% of the cells in a BHK/21 monolayer within a 1-hr virus adsorption period. By taking advantage of this, we were able to construct a single-cycle growth curve for rabies virus and to follow the intracellular course of events by means of fluorescent-antibody staining. The present report summarizes the results of these investigations, and also presents data concerning the mechanism of enhancement of rabies infectivity by polycations.

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MATERIALS AND METHODS

Virus strain. The Pitman-Moore (PM) strain of fixed rabies virus, adapted to growth in human diploid cell strain WI-38 (6) and propagated in these cells for 52 passages, was used to prepare a pool of virus in BHK/21 cells. This pool had an infectivity titer of $10^{5.2}$ to $10^{5.5}$ LD_{50} per 0.03 ml when injected intracerebrally into young-adult (4 to 6 weeks old) mice.

Tissue culture. The following cell lines were used: BHK/21, clone 13 (8); embryonic hamster (Nil-2) (Diamond, *in preparation*); rabbit endothelium (RE) (11); rabbit kidney (RK_{13}) (7); mouse connective

tissue fibroblasts (L, clone 929) (12); rhesus monkey kidney (LLC-MK_2) (4); and a human diploid cell strain, WI-38, derived from embryonic lung (HDCS) (3).

The cells were grown as monolayers in 32-oz prescription bottles in Earle's balanced salt solution containing 0.18% sodium bicarbonate, 10% heat-inactivated calf serum, double concentration of nutrients of Eagle's medium, and chlortetracycline (50 μg/ml). Cells were transferred once or twice weekly, depending on their growth rates, by use of a 0.25% solution of trypsin (Difco) in phosphate-buffered saline (PBS, *p*H 7.4) containing chlortetracycline. The BHK/21 cells were used between the 13th and 22nd passage levels.

Infection of cells. Approximately 1 million cells were inoculated into 60-mm glass petri dishes containing two glass cover slips (11 by 22 mm), and were incubated prior to infection with rabies virus for 24 to 48 hr at 37 C in a humidified atmosphere of 5% CO_2 in air. Subsequently, media from monolayers were removed, and the cells were incubated with 1 ml of virus for 60 min at 37 C. Virus pools were used undiluted; i.e., each cell was exposed to 2 to 5 mouse LD_{50} of virus. The inoculum was then removed, and the cells were washed with 5 ml of warm PBS and reincubated with a fresh 5 ml of growth medium at 37 C.

Added polymers. Stock solutions of the following polyions were prepared in PBS and kept under refrigeration: DEAE dextran, from Pharmacia, Uppsala, Sweden; protamine sulfate (Salmine), from Calbiochem, Los Angeles, Calif.; dextran sulfate 2000, sodium salt, from Pharmacia; heparin, sodium salt, at least 100 units per mg, from Calbiochem. The desired concentrations were made up in virus or tissue culture medium immediately before use.

Immunofluorescent-antibody staining. The direct staining method was used on cover slips from petri dishes in which the monolayers were cultivated. Lyophilized, fluorescein-labeled antirabies globulin (equine) was obtained commercially (BBL), reconstituted, and stored frozen. Just prior to use, PBS was added to give a final 1:30 dilution of the reconstituted material. Technical details of the staining procedure have been described elsewhere (17). Fluorescence observations were carried out by use of a Reichert binocular microscope fitted with UV optics and illuminated with an Osram HBO 200 high-pressure lamp. One thousand cells on each cover slip were counted with the aid of a calibrator in one of the microscope eyepieces. Fluorescent cells were scored, and in some experiments notations were made on the predominant character of the intracellular fluorescing granules according to size (see Fig. 1). This was done because significant quantitative differences within individual cells would not otherwise be apparent in the tabulated results.

Antirabies immune globulin. An antirabies immune globulin preparation was obtained from the Kiev Institute of Epidemiology and Microbiology, U.S.S.R. It had a neutralizing index of 25,000 (i.e., a dilution of 1:25,000 neutralized 10 LD_{50} of the CVS strain of rabies virus injected intracerebrally into mice). The immune globulin had no demonstrable antibodies against LCM virus when tested by complement-fixation, fluorescent-antibody, and serum-neutralization techniques.

UV irradiation. UV irradiation of cultures was performed immediately before inoculation of virus. The growth medium was removed, and the cell monolayers were exposed to a Westinghouse lamp (no. 782–L20) for 5 sec at a distance of 22.5 cm, giving a radiation of 7×10^3 ergs cm^{-2} sec^{-1}.

RESULTS

Effect of polyions on rabies infectivity. Cultures of BHK/21 and HDCS cells were exposed to virus containing a given concentration of DEAE dextran, protamine sulfate, dextran sulfate, or heparin. After incubation at 37 C for 1 hr, the virus media containing polyions were removed, and the cultures were washed and reincubated with normal medium. At intervals thereafter, cover slips were removed and stained for the presence of rabies fluorescent antigen (FA). Table 1 illustrates the effect of the four polyions on rabies infectivity. After exposure to DEAE dextran and protamine sulfate, the percentage of rabies-infected BHK/21 and HDCS cells had markedly increased, whereas the opposite effect was observed after treatment of the cultures with dextran sulfate and heparin. The increase in percentage of FA cells was paralleled by an increase in titer of infectious virus in the culture medium (measured by intracerebral injection into mice).

The enhancing effect of DEAE dextran was further investigated in relation to time of infection. BHK/21 cells were exposed to 50 μg of the compound per ml at the times indicated in Table 2, and the number of cells showing FA 20 hr after infection was determined. The data indicate that pretreatment of cells with DEAE dextran (at -4 and -1 hr) had the same enhancing effect on rabies infectivity as had treatment at the time of adsorption of the virus (0 hr). The polycation had no enhancing effect when added *after* initial exposure to virus ($+1$ hr).

The optimal dose of DEAE dextran causing enhancement was next determined. Although DEAE dextran showed an enhancing effect on rabies infectivity at a concentration of 10 μg/ml, maximal effect was obtained with 50 μg/ml (Fig. 2). Further increase in the concentration of the polymer failed to improve its performance and only increased its toxicity for BHK/21 cells. Treatment of the cells with 50 μg/ml for 1 hr did not show a toxic effect.

In view of the specificity of the enhancing effect of LCM virus in respect to the host cell (16), the action of DEAE dextran was investigated in the different cell systems listed in Table 3. In all systems tested, DEAE dextran was effective in enhancing infectivity. Even with L-929 cells, which are normally insusceptible to rabies infection, the percentage of FA cells rose from less than 0.1 to 6 in 24 hr after treatment with the polycation. The enhancing action was also not

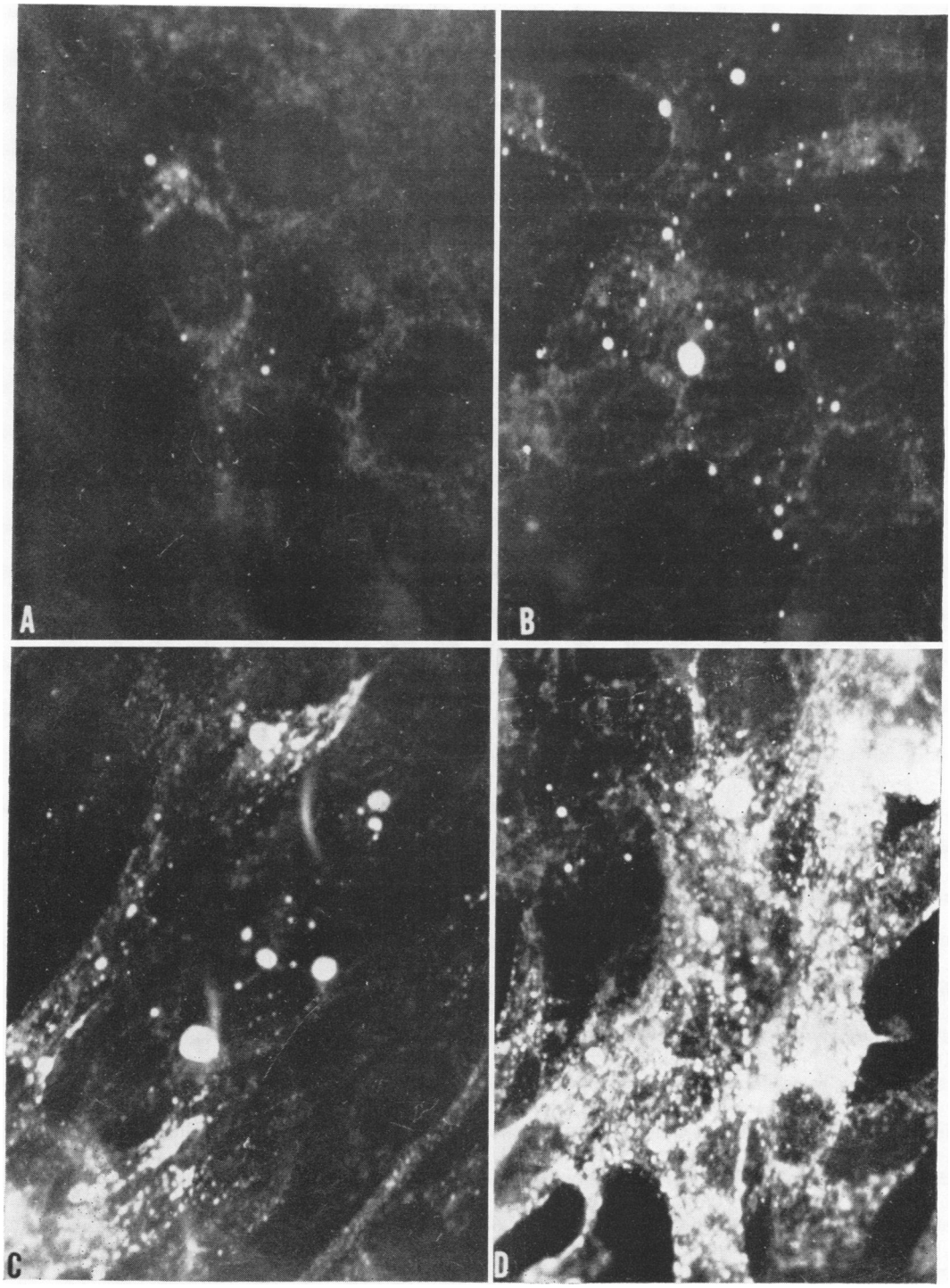

FIG. 1. *Classification of BHK/21 intracellular rabies-specific fluorescent granules according to size. (A) Faint fluorescence, only a few small granules per cell; (B) brighter fluorescence, approximately 10 to 20 small and medium granules per cell; (C) bright fluorescence, more than 50 medium and large granules per cell; (D) bright, coalescent fluorescence, granules large when discernible.*

TABLE 1. *Action of polyions on rabies virus infectivity in BHK/21 and HDCS cells at indicated times after infection*

Polyion	Concn	BHK/21				Per cent FA[a] in HDCS at 72 hr
		20 hr		46 hr		
		Per cent FA[a]	LD_{50}[b]	Per cent FA[a]	LD_{50}[b]	
	μg/ml					
DEAE dextran	50	90	4.0	100	4.8	37
	200	100	4.4	ND	ND	ND
Protamine sulfate	100	55	4.2	100	4.2	24
	1,000	50	ND[c]	90	ND	20
Dextran sulfate	100	3	ND	70	ND	4
	1,000	0.2	<2.0	30	3.2	3
Heparin	500	1	<2.0	1	<2.0	1
Control	—	10	3.4	90	4.0	6

[a] Percentage of cells showing presence of rabies fluorescent antigen (FA).
[b] Negative $\log_{10}$ of virus dilution/0.03 ml killing 50% of mice injected intracerebrally.
[c] Not done.

TABLE 2. *Exposure of BHK/21 cultures to DEAE dextran before and after infection with rabies virus*

Exposure of culture to DEAE dextran (50 μg/ml) in relation of time of infection	FA[a]
hr	%
−4	90
−1	100
0[b]	100
+1[c]	10
No DEAE dextran	10

[a] Percentage of cells showing presence of rabies FA 20 hr after infection.
[b] DEAE dextran added to the virus inoculum and kept during the time of adsorption.
[c] Cells treated with DEAE dextran after removal of virus inoculum.

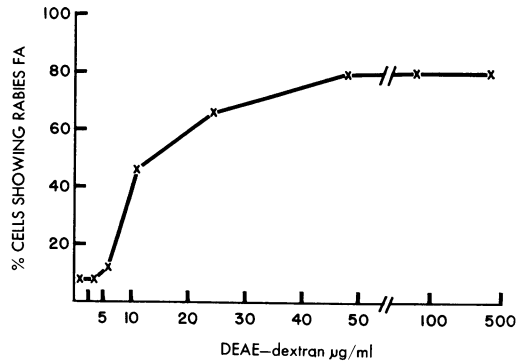

FIG. 2. *Effect of concentration of DEAE dextran on rabies virus infectivity in BHK/21 cells.*

limited to the PM strain of rabies virus; with the CVS strain, and the use of infected mouse brain as the inoculum, the percentage of fluorescent BHK/21 cells in 24 hr increased from 2.5 to 30%.

Effect of rabies antibody on adsorption and penetration of the virus. This problem was studied in BHK/21 cell monolayers exposed to an inoculum of 0.5 ml containing rabies virus and DEAE dextran (50 μg/ml). To this was added antirabies immune globulin (see Materials and Methods) at a final dilution of 1:40 and at the time intervals shown in Table 4. The results indicate that rabies antibody blocked infection completely when added up to 5 min after exposure. When added 5 to 15 min after infection, only 1% of the cells became infected. Antibody had no effect on adsorption and penetration when added to the inoculum 30 min or later after exposure of the cells to the virus. However, spread of virus from the originally infected cells was prevented by the antibody when added to the cultures as late as 60 min after the virus. It should be noted also that lengthening the time of adsorption from 30 to 180 min did not result in an increase in the number of infected cells.

Effect of UV irradiation of cells on their susceptibility to rabies virus. One of the difficulties in constructing a one-step growth curve for rabies virus in BHK/21 cells is that the complete replication time of the virus is longer than the time of division of the cell. Furthermore, rabies-infected cells do not die and can still divide (2). Ways were examined, therefore, of preventing the cells from dividing while maintaining their ability to produce rabies virus. One of these ways, UV irradiation of cell monolayers, was found to make the cells even more susceptible to rabies.

A group of BHK/21 cell monolayers was exposed to UV light for 5 sec, as outlined in Materials and Methods, and immediately afterwards was infected with rabies virus in the presence of

50 μg of DEAE dextran per ml (Table 5). The number of FA cells was determined at frequent intervals in irradiated and nonirradiated cultures, and the concentration of infectious virus in the culture medium was determined at the same time intervals. Observation of irradiated cultures indicated that cells so treated did not divide and usually started to degenerate 72 hr after treatment (Table 5).

The results of the experiment (Table 5) showed that the cells contained rabies antigen 9 hr after infection in both irradiated and nonirradiated cultures, but that the percentage of infected cells was much higher in the irradiated cultures (60%) than in the controls (10%). From then on, the number of FA cells rose rapidly, and the fluorescing granules became larger (CD type; see Fig. 1). The irradiated culture had more FA cells throughout the first 24 hr of infection than the nonirradiated culture, but at 48 hr all cells of both cultures contained rabies antigen. Production of infectious virus was first observed in irradiated cultures 12 hr after infection, as compared with 18 hr in control cultures. The yield of infectious virus was also higher in the UV-treated cultures.

TABLE 3. *Effect of DEAE dextran (DEAE) on rabies virus infectivity in various tissue culture systems*

Cell culture	Per cent FA[a]			
	24 hr		48 hr	
	DEAE absent	DEAE[b] present	DEAE absent	DEAE[b] present
BHK/21	10	100	100	100
Nil-2	5	80	30	100
RE	10	100	30	100
RK_{13}	45	100	50	100
L-929	<0.1	6	<0.1	30
$LLC\text{-}MK_2$	10	39	35	53
HDCS	2	45	5	55

[a] Percentage of cells showing presence of rabies fluorescent antigen at given times after infection.
[b] Concentration, 50 μg/ml.

TABLE 4. *Effect of antirabies immune globulin on penetration and spread of rabies virus in BHK/21 cells*

Time between exposure and treatment with immune globulin	Per cent FA[a]	
	24 hr	48 hr
min		
0.5	0	0
1	0	0
5	0	1
15	0	1
30	50	50
60	60	60
180	55	ND[b]
Control (no globulin added)	60	100

[a] Percentage of cells showing presence of rabies fluorescent antigen at given times after infection.
[b] Not done.

TABLE 5. *Comparative growth of rabies virus in nonirradiated and UV-irradiated BHK/21 cells*

Time after infection	Growth of rabies virus							
	Nonirradiated cells				UV-irradiated cells			
	FA[a]		Mouse LD_{50} ($-\log_{10}$)	Cell count $\times 10^5$	FA[a]		Mouse LD_{50} ($-\log_{10}$)	Cell count $\times 10^5$
	Per cent	Type of granules			Per cent	Type of granules		
hr								
0	—	—	—	8.2	—	—	—	8.2
1	0	—	ND[b]		0	—	2.5	
3	0	—	ND		0	—	2.5	
6	0	—	ND		0	—	2.6	
9	10	A	2.2		60	AB	2.5	
12	10	A	2.0		80	BC	3.5	
15	45	B	2.5		80	CD	3.8	
18	45	B	2.8		80	CD	5.2	
21	45	B	ND		80	CD	4.8	
24	60	BC	4.3	12	85	CD	4.8	5.2
36	80	C	ND		95	CD	4.8	
48	100	CD	ND	27	100	CD	4.8	8.7
60	100	CD	4.0	Deg[c]	100	CD	4.5	Deg[c]

[a] Cells showing presence of rabies fluorescent antigen.
[b] Not done.
[c] Cells degenerating.

One-step growth curve of rabies virus. To assure maximal susceptibility of BHK/21 cell monolayers to infection with the virus for the purpose of constructing a growth curve, it was decided to use UV-irradiated cells exposed to optimal concentrations of DEAE dextran at the time of virus adsorption. The inoculum contained five mouse infective doses per cell which were allowed to adsorb for 1 hr. The cell monolayers were then washed twice with warm PBS and exposed to 3 ml of rabies immune globulin at a dilution of 1:50 in growth medium. The antibody was allowed to act for 1 hr at 37 C, and the monolayers were washed again twice with PBS and incubated in 5 ml of normal growth medium.

At each time interval in Fig. 2, duplicate monolayers were removed, and the percentage of FA cells in one of them was determined. In the other, the medium was removed and frozen, and the cells were washed with warm PBS containing 10% inactivated calf serum. A 5-ml amount of PBS-serum (i.e., a volume equal to the original volume of medium) was then added to the cells, which were frozen and thawed several times and stored at −70 C. The infectious virus content of both the original medium and the washed cells was determined by intracerebral injection into mice.

The results shown in Fig. 3 essentially confirm those obtained with the irradiated cells in the preceding experiment (Table 5). The time of production of the infectious virus could be more exactly determined, however, because the nonadsorbed virus was almost completely neutralized by the antibody.

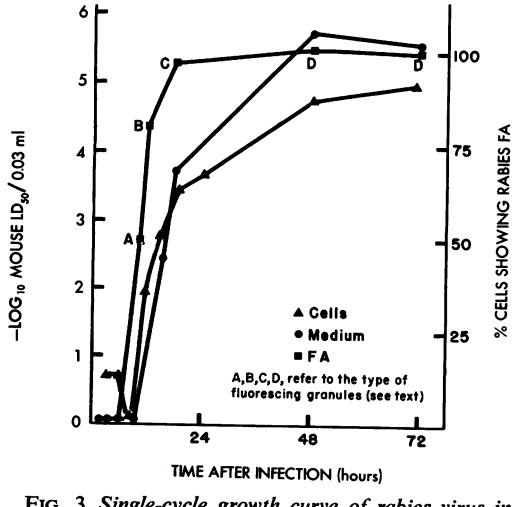

FIG. 3. *Single-cycle growth curve of rabies virus in BHK/21 cell monolayers. Temperature of incubation was 37 C. Input virus multiplicity was 5 mouse* LD_{50} *per cell.*

Presence of rabies antigen was first observed in about 50% of the cells 8 to 9 hr after infection, and there was a further lag period of 3 hr before infectious intracellular virus was produced. Virus was first detected in the medium between 12 and 15 hr after infection. From then on, the number of cells containing rabies antigen and those shedding infectious virus increased rapidly, reaching a plateau (for the FA cells) between 18 and 24 hr after infection. Maximal yield of infectious virus was observed 48 hr after exposure, and at this time more virus was found extracellularly than intracellularly.

DISCUSSION

The action of polyions on rabies virus infectivity appears to be related to their ionic charges: infectivity is enhanced by polymers having a positive charge (DEAE dextran and protamine) and is inhibited by those having a negative charge (dextran sulfate and heparin). Polycations, such as protamine and DEAE dextran, have been used extensively to facilitate the uptake of infectious viral ribonucleic acid by cells in tissue culture (10, 13, and others), but, with a few exceptions, they do not appear to have a corresponding enhancing effect on the infectivity of intact virus. The exceptions include the poliovirus-monkey kidney cell system (10) and the rubella-hamster kidney cell system (Vaheri, Sedwick, and Plotkin, *in preparation*). In the case of mengovirus, evidence has been obtained that protamine *inhibits* virus-cell interaction by binding to cellular receptor sites (1).

The exact site of the enhancing action on rabies virus infectivity by protamine and DEAE dextran has not been determined. The polycations could possibly act by binding to the cellular surface (thereby creating a favorable ionic charge for rabies virus attachment) or by complexing with the virus particles (thereby allowing them to attach to the cell surface more efficiently). Protamine has been reported to precipitate rabies virus (14). It seems likely, therefore, that the enhancing action of this polymer (and, by extension, that of DEAE dextran) involves direct complexing with the virus particles. Whatever the mechanism, however, it is clear that both polycations operate at the very early (adsorption or penetration or both) stages of virus-cell interaction.

By use of optimal concentrations of DEAE dextran and UV-irradiated cells, it was possible to infect 100% of the cells in a BHK/21 monolayer in the 1-hr virus adsorption period. In early attempts to construct a growth curve for rabies virus, immune globulin was not used to reduce residual virus adsorbed to the cells, with the result that high "background" levels of virus were

present throughout the eclipse phase ($10^{2.0}$ to $10^{2.5}$ LD_{50}; see Table 5). With or without the globulin, however, the virus titer seldom rose above $10^{5.5}$ LD_{50} per 0.03 ml. In the absence of DEAE dextran during the adsorption period, the eclipse period was increased by several hours, although the virus titer still eventually reached the same level as it did in the presence of the polycation.

The timing of the first appearance of rabies virus antigen and of intracellular infectious virus corresponds well with the morphological changes occurring in virus-infected cells observed by means of electron microscopy (5). However, it was calculated (Fig. 3) that each infected cell in a BHK/21 monolayer produces, on the average, a maximum of 20 to 30 infectious progeny virus particles, whereas electron micrographs of thin sections of infected cells show that each cell can contain numerous virus particles (5). This would seem to indicate that most of the particles seen under the electron microscope are noninfectious. These apparently noninfectious particles may represent incomplete virus or may be potentially infectious although remaining undetected, e.g., through aggregation or through inefficient methods of separation from cellular material. Indications have been obtained that rabies virus may have to induce synthesis of at least part of its phospholipid components, and it has been suggested that the slow assembly of virus particles may be a direct result of a limited availability of the required phospholipid precursors (9). If this is true, and if the majority of the intracellular virus particles visible in electron micrographs are incomplete because of a lack of essential phospholipid, it should be possible to increase production of rabies virus considerably by determining (and supplying) the missing cofactors or precursors. Work along these lines is in progress.

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