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INVESTIGATIONS ON THE INTERACTIONS BETWEEN POLIOVIRUS RNA AND THE HOST CELL UNDER THE INFLUENCE OF SOME SALT SOLUTIONS

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Summary. — The infectivity of attenuated type 1 poliovirus RNA varies under the influence of certain salt solutions in terms of the nature and concentration of the salts, and reflects the reactivity of the cells treated with these chemical agents.
Although in hypertonic medium the cells incorporate a low amount of RNA, the proportion of highly polymerized molecules is very great. The more accentuated manifestation of viral RNA infectivity in a hypertonic medium appears to be due in the first place to protection of the active molecules against the action of ribonuclease during their penetration into the cells.

Introduction

It is known that, in isotonic media, infective RNA inoculated into cell cultures, exhibit comparatively low infective titres and that treatment of RNA and of the cellular substrate with hypertonic salt solutions represents an efficient procedure for the increase of infectivity (Alexander *et al.*, 1958; Ellem and Colter, 1960; Holland *et al.*, 1960; Koch *et al.*, 1960; Morfei *et al.*, 1967; Popescu *et al.*, 1967; Portocală *et al.*, 1967).

Quantitative investigations with the aid of radioactive isotopes (Borris and Koch, 1964*a*, *b*; Ellem and Colter, 1961; Norman and Veomett, 1961) showed that under the influence of the isotonic medium a higher proportion of RNA is taken up by the cells in comparison to the amount incorporated by the same substrate in a hypertonic medium.

The present investigations were carried out to elucidate the apparently contradictory information concerning the relatively low infectivity of viral RNA under conditions under which its penetration into the cell is favoured, as well as certain aspects of the complex interaction between infective RNA and host cell under the influence of hypertonic salt solutions.

Materials and Methods

Cell cultures. R_1CA (Aderca *et al.*, 1966) and KB cell cultures of the same age (120 hours after seeding into the flasks), maintained under conditions recommended by Aderca *et al.* (1959), were used.

Extraction of infective RNA. RNA was extracted from R_1CA and KB cell cultures infected with attenuated type 1 poliovirus strain Lsc 2 ab (Sabin). The infective titre of the virus after

4 passages was 3.25×10^7 and 4.4×10^6 plaque forming units (PFU) per ml for R_1CA and KB cell cultures, respectively. The cultures were inoculated with 10 PFU of poliovirus per cell. After 6 hours at 37° C, the inoculum was removed, the cultures washed twice with phosphate buffered saline (PBS) without Ca and Mg and the cells were detached from the glass with aqueous 0.01% ethylenediamine tetraacetate (EDTA) solution and 0.15% dodecyl sulphate. The lysate thus obtained was shaken for 5 minutes at 0° C with an equal volume of phenol saturated with water. The aqueous phase obtained by centrifugation for 15 minutes at 2500 rev/min was treated 3 times with ether. RNA was precipitated with 2 volumes of absolute ethanol in the presence of sodium acetate. After 2 hours at —14° C, the RNA precipitate was separated by centrifugation at 2500 rev/min for 40 minutes, dissolved in, and dialysed against distilled water for 2 hours. Approximately 200 μg RNA were extracted from R_1CA cells infected with poliovirus and cultivated in Povitzky flasks (1.4×10^7 cells) and approximately 300 μg RNA were obtained from the corresponding KB cell cultures. The $E_{280} : E_{260}$ ratio of the RNA was 0.44—0.45.

Titration of RNA infectivity was performed in R_1CA and KB cell cultures, subjected to the action of different salt solutions, according to a previously described technique (Portocala *et al.*, 1967). The titres were expressed in PFU/ml values.

Appraisal of cellular viability. R_1CA and KB monolayer cultures were treated under the same conditions as those applied for the titration of infective RNA: twice washed with PBS, once with the salt solution under study and then kept in contact with the same solution for 10 minutes at 37° C. After washing again with PBS the layer was stained for 10 minutes at 37° C with a 0.2% trypan blue solution. The cells were detached from the glass with 0.02% EDTA in PBS and the proportion of dead cells was counted. The control was treated under the same conditions, the salt solution being replaced by PBS.

Isolation of P^{32}-RNA. After removing the growth medium, the R_1CA cell cultures were covered with maintenance medium (97% Earle's solution without phosphates, 0.5% lactalbumin hydrolysate and 2.5% calf serum) to which $Na_2HP^{32}O_4$ (IFA, Bucharest) with an activity of 25 μc/ml medium and 10 PFU of poliovirus/cell were added. The cell monolayers were kept for 6 hours at 37° C and then washed thrice with 150 ml of 0.14 M NaCl, pH 7.4. The cells were detached from the glass and RNA was extracted as described above. After precipitation with ethanol and separation by centrifuging, the RNA was dissolved in 0.005 M Tris [tris (hydroxymethyl) aminomethane] buffer containing 0.05 M NaCl, pH 7.6, treated with 1 μg/ml deoxyribonuclease for 15 minutes at 37° C and again precipitated with 2 volumes ethanol in the presence of sodium acetate. The RNA sedimented by centrifugation was dissolved in distilled water and dialyzed for 3 hours against the same solvent to remove inorganic P^{32}. The specific activity of the RNA obtained was of 200,000 to 250,000 c.p.m./mg, and the $E_{280} : E_{260}$ ratio of 0.44—0.45. After treatment with 5% trichloroacetic acid this RNA was completely precipitable.

Uptake of P^{32}-RNA by the cells under the influence of Na_2SO_4 solutions. The cultures were treated with Na_2SO_4 solutions under the same conditions as those applied for the determination of RNA infectivity. The P^{32}-RNA inoculum (approximately 100,000 c.p.m. for 1.4×10^7 cells) dissolved in Na_2SO_4 solutions of different molar concentrations (Table 2) was kept for 10 minutes at 37° C in contact with the cell monolayer. After washing with PBS, the cells were treated according to the procedure of Borriss and Koch (Borriss and Koch, 1964*a*, *b*; Borriss *et al.*, 1965) with a ribonuclease solution (0.1 μg/ml PBS) for 10 minutes at 37° C, to remove the adsorbed RNA. The monolayers were then repeatedly washed with a 1% bentonite suspension Fraenkel-Conrat *et al.*, 1961). The radioactivity that remained fixed to the cells after these treatments was considered as "absorbed" or "incorporated" RNA. Subsequently, the cells were detached from the glass with a 0.01% EDTA and 0.15% dodecyl sulfate solution. From the homogeneous cell lysates thus obtained, 0.1 ml samples were distributed in aluminium holders to determine the radioactivity with a Geiger-Müller FH 2—15 b counter, with a 1.22 mμ/cm³ frontal window, attached to a Tesla NZQ electronic impulse counter.

The absorbed RNA was analysed by two procedures: a) cold treatment with 5% trichloracetic acid and the measurement of soluble (sTCA) and precipitable (pTCA) radioactivity according to the technique of Amos and Kearns (1963); b) phenol extraction of the incorporated P^{32}-RNA and analysis of the fractions by electrophoresis in agarose gel, followed by autoradiography or recording of the elctrophoregrams (Popa *et al.*, unpublished).

Results and Discussion

The effect of hypertonic salt solutions on RNA infectivity

The system used for testing RNA infectivity under the influence of the factors studied has proved sensitive and reproducible. The relationship between the number of plaques and relative RNA concentrations was linear (Fig. 1).

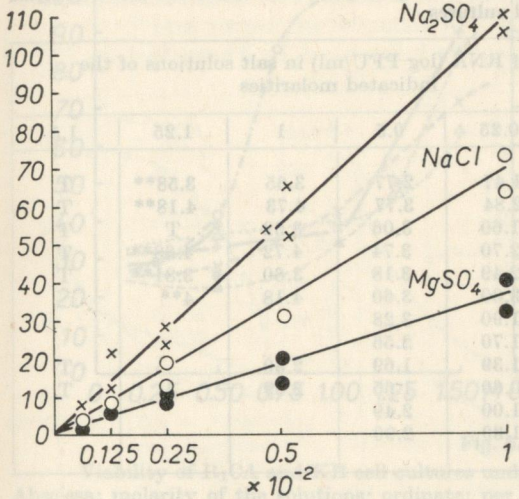

Fig. 1.

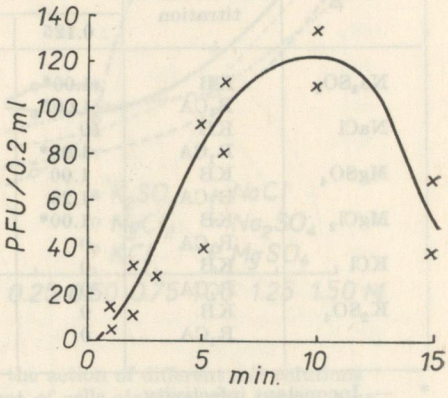

Fig. 2.

Fig. 1.

Infectivity of poliovirus RNA in R_1CA cell cultures under the influence of 1 M salt solutions Abscissa: dilution of RNA; ordinate: total number of plaques formed

Fig. 2.

The kinetics of plaque formation induced in R_1CA cell cultures by RNA treated with 1 M Na_2SO_4 RNA dilution = 10^{-2}.

The kinetics of the appearance of the plaques in terms of the duration of the contact between RNA inoculum and the receptive substrate, was studied for the RNA system in a 1 M Na_2SO_4 solution and R_1CA cells. The graphical representation of this phenomenon (Fig. 2) shows that after a brief latency period, RNA infectivity increases linearly in terms of time up to 10 minutes' contact; then followed a gradual decrease, probably due to diminution of the cellular viability brought about by the toxic effect of 1 M Na_2SO_4. Therefore, under these conditions the 10 minutes' period represents the optimal interval of contact between RNA and the cells, for the correct appraisal of infectivity.

Under the influence of hypertonic solutions, in the conditions described, poliovirus RNA was consistently infective for both types of cell culture. The plaques appearing in R_1CA cultures were round, with a regular contour,

a comparatively homogenous aspect and of variable size (Fig. 3). In the KB cell line the plaques were smaller (Fig. 4).

The experimental data were processed according to the principles of Lorenz (1962). The bilateral significance test was applied to evaluate whether variations in the infective RNA titres were real. With an error probability of 5%, the difference between the maximum titres of the RNA treated

Table 1. The influence of salt solutions on the infectivity of poliovirus RNA for KB and R_1CA cell cultures

Salt	Cell culture used for RNA titration	Titres of RNA (log PFU/ml) in salt solutions of the indicated molarities					
		0.125	0.25	0.5	1	1.25	1.5
Na_2SO_4	KB	1.00*	1.47	2.77	3.65	3.58**	T
	R_1CA	1.00*	2.84	3.77	4.73	4.18**	T
NaCl	KB	0	1.60	3.06	3.32	T	T
	R_1CA	1.00*	2.70	3.74	4.72	4.68**	T
$MgSO_4$	KB	1.00	2.49	3.18	3.80	3.81**	T
	R_1CA	1.26	3.00	3.60	4.18	4**	
$MgCl_2$	KB	1.00*	1.90	3.28			
	R_1CA	0	1.70	2.56			
KCl	KB	0	1.39	1.69	2.50		T
	R_1CA	0	0.60	1.65	2.69		T
K_2SO_4	KB	0	1.00	2.49			
	R_1CA	0	1.30	2.90			

* — Inconstant infectivity
** — Cell layer with signs of partial destruction due to toxic effects.
T — Cell layer destroyed by the toxic effect.
$MgCl_2$ in a 0.75 M concentration caused cell destruction.

with different salts was significant. Insignificant values were obtained only on comparing the titre of the RNA treated with 1 M KCl and that treated with 0.5 M K_2SO_4 and tested on KB cell cultures.

RNA infectivity under the influence of salt solutions in a concentration of 0.125 M was nil or very low (Table 1). Increase in RNA infectivity was directly proportional to the increase in salt concentrations up to a maximum, after which the infectivity values fell or determinations became impossible due to destruction of the cell layer.

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The R_1CA cell line proved to be more susceptible to infection with both the virus and RNA than the KB cell line.

The highest RNA infective titres in the R_1CA cell line were obtained under the influence of 1 M Na_2SO_4, followed in decreasing order by the infectivity values determined under the action of 1 M NaCl, 1 M $MgSO_4$, 0.5 M $MgCl_2$ and 0.5 M K_2SO_4.

The highest RNA infectivity in KB cell cultures was obtained with 1.25 M $MgSO_4$ followed by 1 M Na_2SO_4, 1 M NaCl, 0.5 M $MgCl_2$, 1 M KCl and 0.5 M K_2SO_4.

The relationship between the RNA infective titres determined in certain cell lines by treatment with different salts is not equivalent to that induced by the same factors in other cell lines. It appears that under the influence of the salt solutions RNA infectivity reflects not only the reaction upon

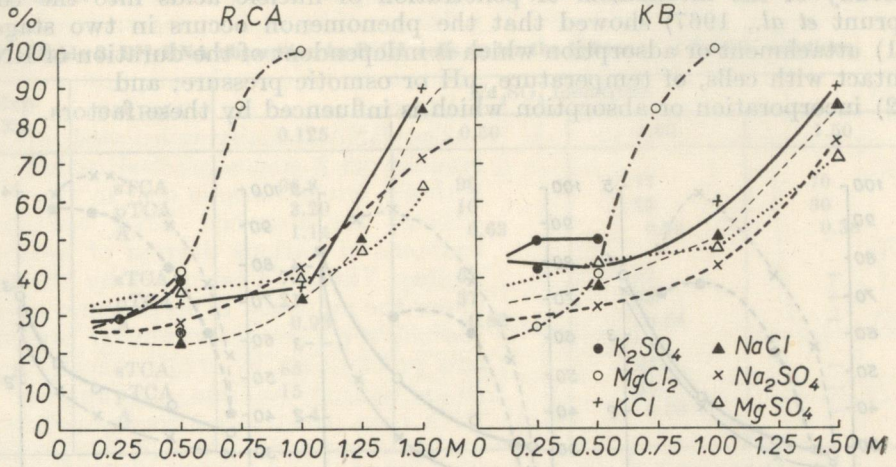

Fig. 5.

Viability of R_1CA and KB cell cultures under the action of different salt solutions
Abscissa: molarity of the solutions; ordinate: per cent of cells staining with trypan blue

the RNA molecule, but also the particular reactivity of different cell species to these chemical agents.

Appraisal of cellular viability

It is obvious that under the action of salt solutions whose concentration varies strongly with respect to the physiological medium of the cells, the biological equilibrium of the latter should be deeply perturbed and that their viability should suffer.

From Fig. 5 it results that KB cells were less resistant to the treatment with salt solutions than the R_1CA cells. This might explain to a certain extent the lower infectivity for KB cells of the viral RNA studied.

The $MgCl_2$ solution was very toxic for both types of cell culture. Up to a concentration of 1 M of the different salt solutions, the mortality rate varied very little. After this limit its increase became very rapid.

The correlation existing between the physiological state of the cells expressed by their viability and viral RNA infectivity is represented graphically in Fig. 6. It is evident that manifestation of the phenomenon of increased RNA infectivity under the influence of hypertonic salt solutions necessitates a cellular substrate whose viability permits multiplication of virus (Ellem and Colter, 1961). A certain degree of damage to the cells, probably reversible, favours manifestation of this phenomenon (Ryser, 1967). In accordance with

the findings of other authors (Ellem and Colter, 1961; Holland *et al.*, 1960; Koch *et al.*, 1960, 1966), the sudden decrease in infectivity at very high molar concentrations coincides with loss of cellular viability.

The uptake of P^{32}-RNA by the cells under the influence of Na_2SO_4 solutions

Study of the mechanism of penetration of nucleic acids into the cells (Sprunt *et al.*, 1967) showed that the phenomenon occurs in two stages:

1) attachment or adsorption which is independent of the duration of RNA contact with cells, of temperature, pH or osmotic pressure; and

2) incorporation or absorption which is influenced by these factors.

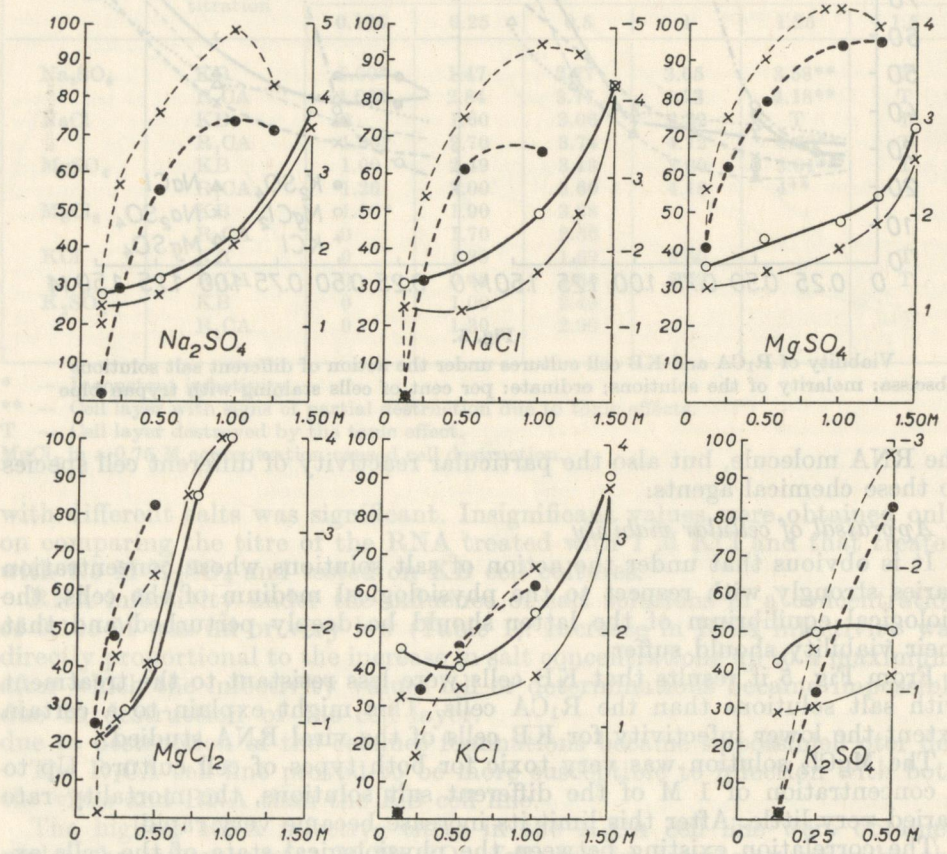

Fig. 6.

Relationship between poliovirus RNA infectivity and cell viability under the influence of Na_2SO_4, NaCl, $MgSO_4$, $MgCl_2$, KCl and K_2SO_4 solutions

Abscissa: molarity of the solutions

Left ordinates: per cent of KB (○———○) and R_1CA (×———×) cells stained with trypan blue

Right ordinates: RNA infectivity in log PFU/ml values for KB (●------●) and R_1CA (×------×) cell cultures

Attachment of RNA to the cellular surface is reversible in the first phase. After a period that varies in terms of the system investigated and the methods applied, adsorption becomes irreversible.

From the total amount of attached RNA only a small proportion, whose value varies with the experimental conditions, is absorbed by the cells (Borriss *et al.*, 1965; Ellem and Colter, 1961; Norman and Veomett, 1961).

Table 2. P^{32}-RNA absorption in R_1CA cells under the influence of Na_2SO_4 solutions

Exp. No.	P^{32}-RNA	Na_2SO_4 molarities			
		0.125	0.50	1.00	1.50
1	sTCA	96.8	90	77	70
	pTCA	3.20	10	23	30
	A	1.14	0.63	0.39	0.38
2	sTCA	79	63	57	—
	pTCA	21	37	43	—
	A	0.96	0.82	0.64	—
3	sTCA	85	—	65	—
	pTCA	15	—	35	—
	A	1.4	—	0.70	—

sTCA and pTCA = Proportion (%) of RNA soluble in, or precipitated by trichloroacetic acid, respectively, from the total amount of radioactivity absorbed by 1.4×10^7 R_1CA cells.

A = Total proportion (%) of absorbed radioactivity referred to the amount of radioactivity inoculated into each monolayer of 1.4×10^7 R_1CA cells.

— = Not done.

Most workers that have studied this process (Borriss and Koch, 1964a; Ellem and Colter, 1961; Norman and Veomett, 1961) assert that, in isotonic media, the amount of viral or other RNA adsorbed on the cells is greater than that attached under the influence of hypertonic media.

These data are in contradiction with the results obtained in studies on the infectivity of viral RNAs. To explain this discrepancy, several working hypotheses may be proposed:

a) although the amount of RNA attached to the cells in isotonic media is greater, nucleic acid absorption is smaller than that occurring in a hypertonic medium;

b) the structural integrity of the incorporated RNA molecule is affected to a greater extent in media with physiological salt solutions than in media with higher salt concentrations.

In our investigations on RNA absorption by the cells under the influence of different Na_2SO_4 concentrations, we attempted to establish the amount of RNA taken up by the R_1CA cell monolayer and the degree of hydrolysis of this RNA. The results are presented in Table 2. From an equal amount of RNA added to each monolayer, a very small proportion ranging from 0.38 to 1.4% was absorbed by the cells. These values were very close to

those of Ellem and Colter (1961) and lower than those of Borriss and Koch (1964*a*) and Borriss *et al.* (1963) obtained under different experimental conditions.

Although the values varied from one experiment to another with respect to different factors, it appears obvious that a larger amount of P^{32}-RNA was incorporated by the cells under the influence of low salt concentrations. Increase in the molar concentration brought about a decrease in the amount of RNA taken up.

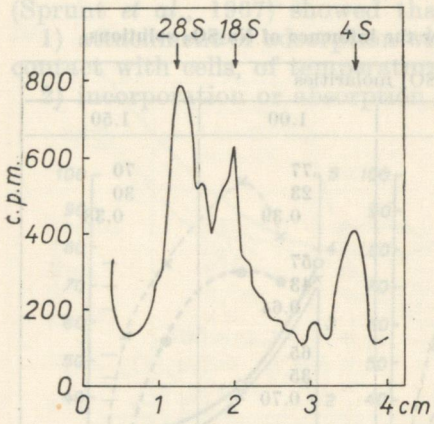

Fig. 7.
Electrophoregram of P^{32}-RNA before inoculation in R_1CA cell cultures
Abscissa: distance from start in cm; ordinate: radioactivity (counts per minute)

The RNA initially inoculated in the monolayers was precipitated completely with 5% trichloroacetic acid in the cold, whereas the absorbed RNA contained fractions of various degrees of acidosolubility. The RNA taken up by the cells under the influence of hypertonic media showed a higher proportion of components precipitable by trichloroacetic acid. In an isotonic medium, the amount of RNA that penetrates into the cells was mostly represented by acid-soluble, therefore hydrolysed, RNA.

The P^{32}-RNA absorbed by R_1CA cell monolayers under the influence of different Na_2SO_4 solutions, was also analysed by electrophoresis of the RNA samples in agarose gel. The relative value of the RNA fractions separated by electrophoresis was determined by autoradiography. Fig. 7 presents the electrophoregram of the RNA initially inoculated into the cell monolayers. This product contained three main fractions: two ribosomal (28 S and 18 S) and a soluble (4 S) RNA.

Fig. 8 presents the autoradiographs of the P^{32}-RNA incorporated by the cell in a medium with 0.125 M and 1 M Na_2SO_4. Under the influence of the 0.125 M solution the absorbed RNA showed very small fractions in the 28 S and 18 S regions and a high amount of a fraction with a molecular weight of about 4 S. In contrast, under the influence of 1 M Na_2SO_4, a large amount of highly polymerized RNA penetrated into the cells. It may be asserted that, although the total amount of RNA that had penetrated

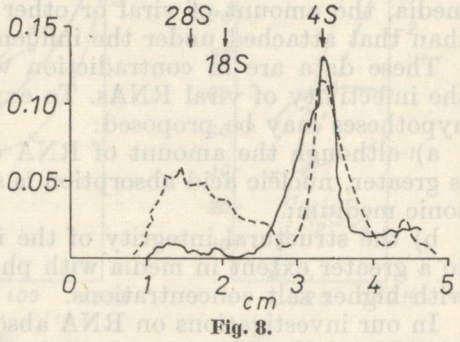

Fig. 8.
Electrophoresis of P^{32}-RNA incorporated into R_1CA cell cultures under the influence of 0.125 M Na_2SO_4 (———) and 1 M Na_2SO_4 (- - - - -)
Abscissa: distance from start in cm; ordinate: O.D. at 510 nm.

into the cells under the action of a hypertonic medium was comparatively small, the proportion of highly polymerized macromolecules was high.

The apparent contradiction between potentiation of RNA infectivity in media with a high salt concentration and the low uptake of RNA under the same conditions is thus explicable. Hypertonic salt solutions may favour infection of the cells with viral RNA by protecting this RNA against the hydrolytic action of ribonuclease, either on the surface of the cell or within it, and not by increasing the degree of RNA adsorption onto the respective substrate.

It may likewise be assumed that in media with a high salt concentration the process of preferential adsorption of the highly polymerized molecules reported by Borriss and Koch (1964*a*) is intensified.

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