Factors Influencing the Infectivity of Poliovirus Ribonucleic Acid.* (27057)

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III. *Role of Host Cell in Determining Infectivity of Ribonucleic Acid Under Varying Conditions.* The nature of the change responsible for loss of infectivity of free poliovirus ribonucleic acid (RNA) in the presence of isotonic salt solution and complete recovery when salt concentration is increased(1) is of interest in a number of disciplines. Fraenkel-Conrat(2), Doty and others(3) have reported that a structural change in the RNA molecule occurs with changes in salt concentration. This paper presents data which indicate that modification of the host cell also occurs with changes in salt concentration. Further, host cell susceptibility to RNA infectivity varies with increasing sucrose concentration, age of cell, nutrition factors during growth and temperature during invasion by RNA.

Materials and Methods. Cell lines. Amnion cells (Fernandes line(4)), HeLa cells (a cloned line selected in this laboratory for susceptibility to polioviruses(5)), and HEP_2 cells were grown for a week in 32 oz prescription bottles, then were treated with trypsin and transferred to 60 mm Petri dishes for growth of monolayers.

Media. Unless otherwise stated, all cell lines were grown in bottles and plates in a modified Eagle's solution made up with double the usual amounts of amino acids and vitamins.

RNA. RNA was prepared by phenol treatment of type 1 poliovirus (Mahoney strain) grown in tissue culture(6,7).

Inoculation of monolayers for measuring degree of infectivity by plaque counts. Before inoculation, cells were washed twice with isotonic salt solution buffered with Tris to pH 8. The inoculum of 0.1 ml of RNA preparation was left on the plates for 30 minutes at room temperature before nutrient agar layering, unless the routine was varied, as stated in the text. In some instances, where the inoculum was considered traumatic or adsorption time was long, the cells were washed once with isotonic salt solution before layering with nutrient agar. Details of media and technique were described previously(7).

Adsorption time. The term "adsorption time" is used in this paper to indicate the interval between inoculation of RNA and layering with agar or serum-containing solution to inactivate free RNA.

Inactivation of RNA and whole virus. In experiments measuring adsorption time, RNA was inactivated at the time indicated by layering the plates with 2 ml of buffered isotonic salt containing 10% horse serum or normal monkey serum. Whole virus was inactivated similarly with a solution containing 10% anti-type 1 polio monkey serum.

Experimental Results. The Salt Effect. Influence of salt concentration on infectivity of polio RNA in 3 cell lines. To differentiate the effect of salt on the RNA molecule from the effect of salt on the host cell, infectivity of the same RNA preparation was tested simultaneously in amnion, HeLa and HEP_2 cells. The cells in monolayers were inoculated with RNA diluted in the concentrations of NaCl and KCl noted in Fig. 1, and susceptibility was determined by counting the number of plaques which appeared under the conditions described. Fig. 1, presenting composite results from 5 experiments, indicates that amnion and HeLa cells are similar in relative susceptibility to RNA suspended in different concentrations of NaCl and KCl. Uniformly, sodium ions are more effective in enhancing infectivity at lower molarities than potassium ions. The 2 lines respond quite differently, however, to RNA diluted in increasing concentrations of both salts. HeLa cells are more susceptible to RNA at salt concentrations below 1 M than amnion cells, but titer is little enhanced with increase in salt concentration beyond this point. On the other hand, amnion cell susceptibility to RNA infection is roughly proportional to the salt concentration of either diluent until the limit of cell tolerance for salt

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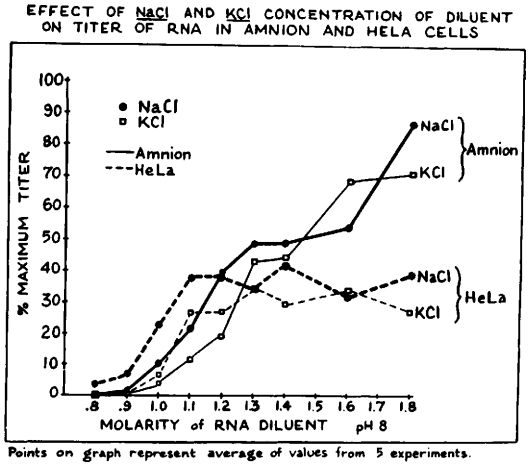
is reached at 1.8 to 2 M. Subsequent tests with HEP_2 cells carried out with NaCl alone indicate that HEP_2 cells are the most sensitive of the 3 to the influence of this salt. RNA infectivity titers in HEP_2 cells at low molar-



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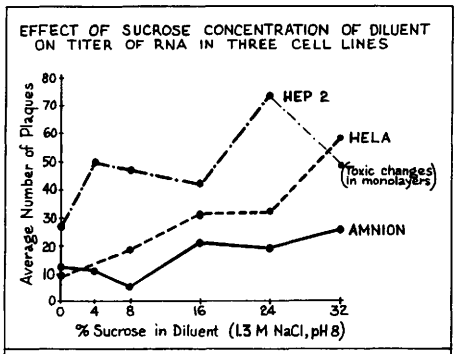

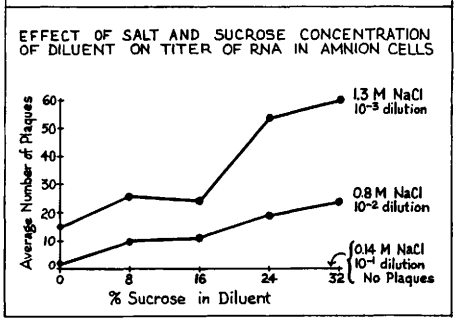

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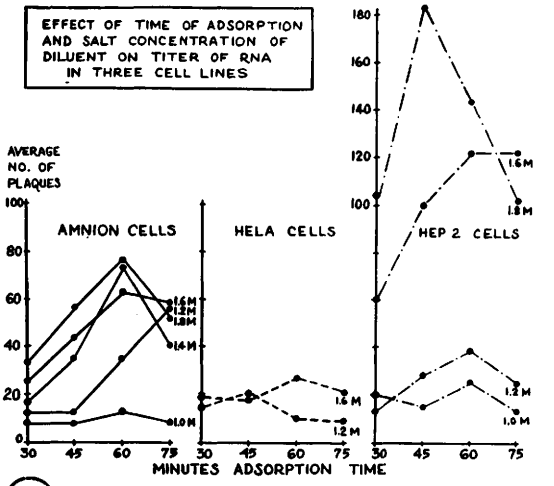

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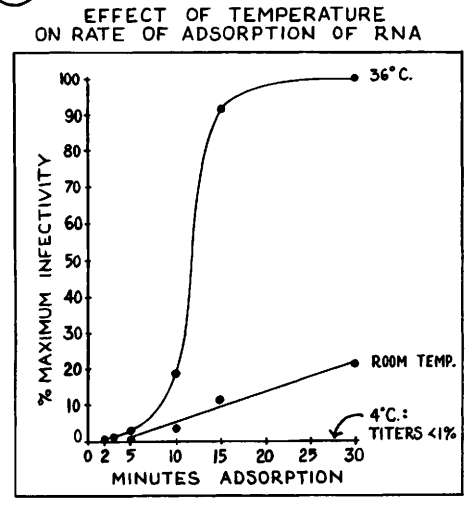

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FIG. 1-4

ities are higher than those of HeLa cells, and at higher molarities there is a titer increase proportional to increase in salt concentration similar to that in amnion cells but the increments continue at a higher level.

The titer of whole virus is not significantly different as measured in the 3 cell lines and is not influenced by the salt concentrations used in the experiments on RNA.

Influence of salt concentration and time of adsorption on infectivity of poliovirus RNA in 3 cell lines. The same effect of cell line was demonstrated in experiments which tested prolongation of adsorption time at varying salt

concentrations. In amnion and HEP_2 cells, there was a significant increase in degree of infectivity of RNA with time between 30 and 60 minutes after seeding and with increase in salt concentration above 1 M. In HeLa cells no increase in infectivity occurred with adsorption times longer than 30 minutes, and there was no significant enhancement with salt concentrations above 1 M. These experiments were carried out in the usual fashion except that the cells were washed once with isotonic salt solution containing 10% normal serum (monkey or horse) before being layered with agar. Fig. 2 gives the data from one of 3 such experiments, all of which showed quite similar results. The most notable variable was an increase of RNA infectivity with increase in adsorption time in one of the 3 experiments with 1.2 M diluent in HEP_2 cells and in 2 of the 3 in amnion cells. It is apparent that 1.2 M NaCl is a borderline concentration between the 1.0 M range to which there was uniformly no titer increase with adsorption time longer than 30 minutes and the 1.4 M range in which such titer increase was regularly reproducible.

There is relatively little effect of salt and these increments of adsorption time on titer of whole virus under similar circumstances. This suggests that the differential susceptibilities of the different cell lines to RNA infectivity are more than a measure of the ability of the cell lines to grow poliovirus in the presence of unusual concentrations of salt.

Influence of sucrose concentration on infectivity of polio RNA in 3 cell lines. The effect of sucrose on RNA infectivity was studied to see if there was an additional increase in infectivity due to increasing osmolarity without increasing salt concentration. Again, it was evident that HEP_2 cells showed the greatest sensitivity to altered environment with a definite rise in titer of RNA infectivity with increasing sucrose concentrations up to the point of cell toxicity (usually at 35-45%). The response of HeLa and amnion cells was less regular, but in general HeLa cells seemed a little more sensitive to increasing sucrose concentrations. Increase in RNA infectivity with increase in sucrose seemed relatively greater at higher salt concentrations. This proved to be true in about the degree shown in Fig. 3, in 3 out of 4 experiments. We have not been able to demonstrate infectivity of RNA in isotonic diluent with added sucrose up to 32%. This is in contrast with the findings of Ellem and Colter(8) who found their highest titers with RNA in isotonic salt solution plus added sucrose to 17%.

Influence of preliminary treatment of host cells with solutions of high pH and salt concentrations. To explore the direct effect of salt concentration on the cells in promoting enhanced RNA infectivity, amnion cell monolayers were washed before inoculation with fluids containing varying salt concentrations. The wash fluids were removed as completely as possible before seeding with RNA in either isotonic or hypertonic salt diluents. The results of a typical experiment are shown in Table 1. Preliminary washing with hypertonic saline at pH 8 increased infectivity only if the RNA diluent was suboptimal in molarity and pH.

RNA in isotonic diluent regularly showed a low degree of infectivity following high salt wash however thoroughly the wash fluid was drained from the cell layer (Table 1). Under these circumstances, the only plaques which formed showed such a regular pattern of distribution (either limited to the peripheral gutter of the plate or to a halfmoon area extending from the center of the plate to the gutter) that effect of the residual salt on the RNA molecule rather than on the cell surface could not be ruled out. The localized plaque pattern still formed when the cells were preincubated for 10 or more minutes in a 1.6 M NaCl solution which was thoroughly removed immediately before inoculation. No plaques appeared at all when such pretreated cells were quickly flushed with isotonic solution and inoculated immediately thereafter. Preliminary "conditioning" of the cells with hypertonic salt apparently had no measurable lasting effect on ability of the cells to become infected by RNA in low salt concentrations. Since the "salt effect" on infectivity is known to be very rapid, however, the lack of cell "conditioning" with salt is not decisive in either direction.

Alteration of susceptibility of host cell to RNA by factors other than salt concentration. The influence of factors other than salt con-

TABLE I. Effect of Salt Concentration and pH of Solutions used to wash Amnion Cell Monolayers before Inoculation on Infectivity of RNA Diluted in 0.14 M or 1.3 M NaCl at pH 8.

Salt Concentration of Wash Solution	Number Plaques Produced by RNA Diluted in Salt Concentration Noted and Tested on Monolayers Washed with pH Noted.			
	pH 7.2		pH 8.0	
	.14 M (10^{-2} dil)	1.3 M ($10^{-3}/_2$ dil)	.14 M (10^{-2} dil)	1.3 M ($10^{-3}/_2$ dil)
.14 M	0	32*	0	30
.6 M	.5	28	.7	35
.8 M	2.6	21	3.8	35
1.0 M	11.0	21	16.0	31
1.1 M	14.0	27†	20.0	27
1.3 M	31.0	16†	48.0	30

* Avg number plaques/3 plates. † Toxic changes in monolayer.

centration on degree of infectivity of polio RNA offers additional evidence in favor of the importance of the state of the host cell on degree of infectivity expressed by an RNA.

Effect of age of cells at time of inoculation on susceptibility of the cells to RNA. Within the time limits, 1 to 4 days, age of cells had no effect on cell susceptibility to whole virus. Table 2 shows, however, that monolayers of both amnion and HEP_2 cells grown for one day were clearly less sensitive to RNA than cell layers incubated for 2 to 4 days prior to inoculation.

Effect of environment of cells during growth on their susceptibility to RNA. Experiments which used the 2 M $MgSO_4$ diluent and calf serum fed monolayers recommended by Holland *et al.*(9) provided an interesting illustration of the effect of cell environment during growth on susceptibility of the cell to infection by the same RNA diluted in 2 M $MgSO_4$ or in 1.6 M NaCl. These experiments were unusual in that the cells were refed with the feeding solutions indicated in Table 3 twenty-four hours before inoculation, were washed with isotonic solutions after the adsorption period and were layered with agar containing the same serum with which they had been fed originally. Serum concentration was the same throughout except in the "not refed" plates where the final agar overlay contained 10% serum instead of the 20% used in the others. Table 3 presents data averaged from 6 experiments indicating that cell feeding solution had no significant effect when RNA was suspended in NaCl but that there was a marked effect of cell propagating fluid on measure of infectivity of the same RNA suspended in $MgSO_4$. Again, it seems likely that

TABLE II. Effect of Age of Monolayer on Titer of RNA.

Age of Monolayers (Days)	Amnion Cells Exp. No.			HEP_2 Cells Exp. No.		
	1*	2	3	1*	2	3
1	4**	5	7	14	23	25
2	10	31	18	36	62	62
3	21	53	30	24	58	69
4	12	15	26	31	61	88

* Each experiment done simultaneously in the 2 cell lines.

** Avg number of plaques on 4 monolayers.

TABLE III. Effect of Environment of 3 Cell Lines During Growth on Susceptibility to RNA Diluted in 1.6 M NaCl and 2 M $MgSO_4$.

Growth Medium	Plaque Count Produced by RNA Diluted in Following Salt Solutions and Tested in 3 Cell Lines.					
Monolayers refed 24 hrs. before inoc. with Eagle's Soln. + 20%	Amnion Cells		HeLa Cells		HEP_2 Cells	
	NaCl	$MgSO_4$	NaCl	$MgSO_4$	NaCl	$MgSO_4$
Calf Serum	12*	23	33	31	24	27
Horse "	18	12	29	22	29	14
Human "	12	13	28	12	24	11
Not Refed	11	4	23	4	26	8

* Plaque count averaged from 6 exp. 3 or 4 plates per lot per exp.

the physiological state of the host cell plays an important role in its susceptibility to infection by RNA.

Effect of temperature on uptake of RNA is considered here because it indicates that the host cells probably play an active role in the process of RNA uptake. Fig. 4 shows the marked effect of temperature on degree of infectivity of RNA following exposure to cells for various intervals at 4°C, room temperature, and 36°. This effect on RNA was somewhat similar to but different in rate and more exaggerated in degree than that on whole virus under the same conditions. Amnion cells were used in the experiment shown. All equipment, the cells, wash solutions and RNA were at the temperature indicated before and throughout the experiment.

Infectivity was maximal in the time interval shown at 36°C, reduced at 25°C, and at 4°C usually less than 1% of that found at 36°. The marked reduction in infectivity at 4°C could be due to diminished molecular mobility and hence diminished chance of contact of RNA molecules with cell surfaces. Against this explanation, however, is the fact that constant machine agitation of monolayers for 45 minutes after seeding did not increase infectivity at 4°C significantly. It seems likely that the process of RNA infection requires metabolic activity on the part of the host cell which is inhibited at low temperatures.

Discussion. Doty and his associates(3), and others before them, showed that the structural configuration of the RNA molecule changes with change in salt concentration, kinking up in high molarities and straightening out and perhaps dissociating in lower molarities and in water. While such alteration in the polio RNA molecule may play a role in its infectivity in tissue culture, the evidence presented here indicates that the effect of hypertonic salt environment on infectivity is at least in part the result of changes in the host cell. In the experiments described in the first part of this paper, the RNA molecule may have changed with the changes in salt concentration, but the influence of salt concentration on degree of infectivity of the same RNA preparation (plaque count) varies in different cell lines.

The data presented in the second part of the paper emphasize that a number of factors in addition to salt concentration may alter cell reaction to RNA to an important degree. It seems likely that such factors as those described and others as yet unknown are playing an important role in producing the variations sometimes found in attempting accurate quantitative work with RNA. Ordinarily, individual monolayers inoculated with RNA measure infectivity as reproducibly as monolayers inoculated with whole virus and relatively little variation in counts is found among plates receiving the same inoculum. From time to time, however, plate to plate variation seems unaccountably large and increasing numbers of plates must be used to achieve overall reproducibility of results. It seems likely that the unexplained variability is due to a variation in the state of the host cell, regardless of all attempts to keep the cell state as constant as possible. To obtain maximal plaque counts, RNA infectivity experiments have to be carried out under conditions visibly damaging to the host cell, and it is logical to assume that the irreversibility of such damage and resultant inability of the cells to support growth of virus will be variable, especially when the cells are not in optimum condition. For this reason, we do not use any of the diluents described here at their maximum concentration tolerated (1.8 M NaCl, 40%+ sucrose, etc.). A diluent of 1.6 to 1.7 M NaCl at pH 8.5 with or without sucrose to 24% gives more reproducible results. Even with all precautions and despite the fact that individual tests with RNA usually look reliable, it is our experience that experiments should be repeated several times to be sure of the dependability of the results. In our hands, at least, measurement of RNA infectivity is not yet as quantitatively reliable as measurement of whole virus infectivity in the same cell system.

It might be said that the experiments described here measure only the ability of the cells to grow poliovirus under the circumstances described. This is not a tenable explanation, however. Whole virus was tested in parallel with most of the foregoing experiments, and the differences described in changing the ability of the cell lines to detect RNA

infectivity were not demonstrable for whole virus.

Summary. Evidence presented shows that the influence of salt concentration of the environment on RNA infectivity for mammalian cells is due at least in part to alteration of the state of susceptibility of the host cell. The 3 different cell lines tested simultaneously responded differently when exposed to the same RNA preparation diluted in varying salt concentrations. Other factors which alter host cell susceptibility, sucrose concentration, age of host cell, nutritional environment of host cell and temperature during the adsorption interval, emphasize the importance of the physiological state of the cell at time of exposure to RNA to the degree of infectivity expressed.

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Failure of Reserpine to Cause Depletion of Gastrin in Canine Antral Mucosa.‡ (27058)

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(Introduced by F. J. Stare)

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Although the chemical composition of the hormone gastrin is not known, it is thought to be a protein-like substance of low molecular weight, or possibly a more simple substance attached to a protein molecule. Gastric mucosa is known to contain both histamine(1) and serotonin(2) and these have been considered as possible constituents of gastrin.

Reserpine has the property of stimulating acid secretion from the stomach(3). One possible mechanism for this action is the release of gastrin from the antral mucosa. Reserpine is known to deplete tissue of catechol amines(4), serotonin(5) and histamine(6), and if gastrin were bound in the tissue by the same mechanism as those amines, then reserpine might be expected to release gastrin also.

If the effect of reserpine upon gastric secretion is exerted through release of gastrin, it might be expected that the gastrin content of the antral mucosa would be reduced following its administration. Indirect evidence concerning the storage and binding of gastrin might also be established in this manner.

Methods. Reserpine was administered by mouth in a dose of 5 mg per kg body weight to 13 adult mongrel dogs. Twenty-four hours later the animals were killed and the stomachs immediately removed. Each stomach was opened, washed and mucosa of the antral region dissected from the underlying muscle. The mucosa was stored at $-70°C$ until required.

Extraction. The mucosa was thawed and minced and extraction was performed by the

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