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Factors Influencing Degree of Infectivity of Enterovirus Ribonucleic Acid.* (25499)

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I. *Reversibility of Infectivity of Poliovirus Ribonucleic Acid by Changes in Salt Concentration.* A number of investigators found that free ribonucleic acid (RNA) obtained from several different viruses exhibits only approximately 1/1,000th of the infectivity of intact virus from which it is derived. Fraenkel-Conrat(1) suggested that residual ribonuclease (RNAase) activity in his Tobacco Mosaic Virus RNA preparations accounts for at least some of the apparent instability of RNA, and that RNAase activity is particularly marked in isotonic solutions. Koch *et al.*(2) suggested that the lower infectivity of RNA as compared to whole virus might be due partly to a different and probably less efficient mechanism of entry of RNA into the cell. Our experiments were carried out to gain an understanding of factors which influence degree of infectivity of the active portion of RNA. Such information may be expected to throw some light on the explanation of the differential between infectivity titer of whole virus and that of RNA derived from it. This paper presents evidence that loss of infectivity of poliovirus RNA which occurs on dilution in isotonic salt solutions can be recovered quantitatively by addition of salt (NaCl and KCl).

Materials and methods. Infectivity test system. The cell line used throughout was the Fernandes passage line of human amnion cells (3) grown in Eagle's solution with twice the recommended concentration of amino acids and vitamins(4). RNA was inoculated on 3 day-old cells grown on 60 mm Petri dishes, and the plaque count was made 4 days later. Prior to inoculation with RNA the cells were washed 2 x with isotonic solutions. In all respects, other than use of isotonic wash solutions, the methods were identical to those described previously(5). *Solutions used.* "Minus" Hanks Tris (–HT) consisted of Hanks solution minus calcium, magnesium and phosphate, and buffered at pH 8 with Tris buffer. D_3T = 0.9 M NaCl, 8% sucrose, plus 0.1 M Tris buffer adjusted to pH 8 with HCl. E_1 virus is a preparation of partially purified (100 x concentrated) type 1 poliovirus received from Cutter Labs. It is said to have a molarity of approximately 0.88 with respect to NaCl. Infectious RNA was prepared by modification of phenol extraction method of Gierer and Schramm(6). This method has been described(5). *Alcohol Precipitation.* One part RNA was mixed with 2 parts 95% ethanol and kept at 4°C overnight. Usually no visible precipitation occurred, but since infectivity is regularly quantitatively recovered there seems reason to believe that RNA is precipitated by this process and adheres to the glass. The mixture was then centrifuged in a cold centrifuge at approximately 8,000 rpm for 30 minutes. The supernatant was discarded and glass-distilled water added to the residue in volume equal to that of original RNA preparation.

Results. An RNA preparation which has lost its infectivity in isotonic salt solution can regain its full degree of infectivity when diluted further in hypertonic salt solutions. Table I compares degree of infectivity of the same RNA preparation diluted in solutions of 3 different salt concentrations at constant pH. Two different RNA preparations were exam-

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TABLE I. Effect of Molarity of Diluent on Degree of Infectivity of Ribonucleic Acid.

Origin of RNA	10^{-1} dilution made in	No. of plaques per plate, 10^{-1} dilution	Titer* on dilution in .9 M KCl + Tris	Titer* on dilution in .14 M –HT†
E_1‡ (.88 M)	.9 M KCl-Tris		1.9×10^4	0
	.14 M –HT		8×10^3	0
	D.W.§		1.3×10^4	0
E_1‡ (.88 M) Fresh RNA	.9 M KCl-Tris		1.6×10^4	
	D.W.	0, 0, 0	5×10^3	0
Frozen RNA	.9 M KCl-Tris		4×10^3	
	D.W.	0, 0, 2	4×10^3	0
Isotonic virus‖ (.14 M)	.9 M D_3T		approx. 5×10^5 (C)¶	
	.14 M –HT	0, 1, 0, 0	4.5×10^5 (C)	
	D.W.	0, 0, 0, 3	3.5×10^5 (C)	

* Plaque-producing particles/ml.

† –HT = Hanks Solution minus calcium, magnesium, phosphate + Tris buffer.

‡ E_1 fraction = Partially purified 100 × concentrated preparation of type 1 poliovirus (Mahoney strain), received from Cutter Labs; said to be .88 M with respect to NaCl.

§ D.W. = Glass-distilled water.

‖ Mahoney strain type 1 poliovirus grown in tissue culture in Scherer's Maintenance Solution + 10% horse serum; probably approximately .14 M.

¶ (C) = Confluent areas—counts partly estimated.

ined, one derived from the E_1 fraction of partially purified 100 x concentrated poliovirus, and one from crude tissue culture virus in isotonic media. Each was prepared by procedure described previously(5) and number of plaques/0.1 ml was measured after 10^{-1} dilution in 0.9 M KCl buffered with Tris,† 0.14 M "minus" Hanks solution (–HT) and glass-distilled water, all with pH approximately 8. These 10^{-1} dilutions of each RNA in the 3 different salt concentrations were further diluted to final concentration of 10^{-2} in 0.9 M KCl and Tris, and 0.14 M –HT and examined for plaque count. Table I shows that E_1 RNA, freshly prepared or frozen, or RNA from crude tissue culture virus in isotonic solution, if diluted 10^{-1} in distilled water or 0.14 M –HT saline only occasionally produces a plaque. However, when this non-infectious RNA is further diluted in hypertonic salt solution the original degree of infectivity can again be demonstrated.

In Table II are listed results of experiments on the influence of duration of exposure to hypotonic or isotonic environments on degree of recovery of infectivity when RNA so exposed is transferred to hypertonic salt solutions. The results listed in Table I were derived from experiments which had been carried out within a few minutes, and left open the possibility that irreversible damage to the RNA molecule might occur in hypotonic solutions if exposed for longer periods of time.

In spite of the long periods of exposure to isotonic or hypotonic environments, recovery of infectivity occurred regularly in hypertonic solutions. There is suggestive evidence that recovery was not always complete.

To reduce salt concentration to the lowest feasible degree and to eliminate the variable of small changes in salt concentration which existed in experiments in Table II (0.87 M experimental dilutions and 0.9 M controls), an RNA preparation made from a suspension of virus in isotonic medium was precipitated with alcohol, resuspended in distilled water, then exposed to distilled water in 10^{-1} dilution for periods varying from 1 to 24 hours. Low concentrations of salt were presumably still present but were probably less than 0.001 M. At each period shown in Table III the RNA was further diluted in D_3T so that final inoculum contained 0.9 M NaCl. Immediately and 60 minutes later, 0.1 ml was seeded on each of a series of monolayers. Where the difference in molarity between test and control RNA has been eliminated, no evidence was found of differences in degree of recovery of infectivity

† The 10^{-1} dilution in 0.9 M KCl buffered with Tris was not tested. Previous experience showed that this dilution regularly produced confluent cytopathogenic action.

TABLE II. Effect of Time Interval in Isotonic Solution on Titer of Ribonucleic Acid Further Diluted in Hypertonic Diluent.

Origin of RNA	Time diluted 10^{-1} in D.W.	No. of plaques in D.W.	Titer* on dilution in .9 M D_3T† Stat	30 min.	60 min.
E_1 (.88 M)	1 hr	0, 0	6.5×10^3		8.5×10^3
	3 "	0, 0, 0		4×10^3	7×10^3
	5 "	0, 0, 0		6×10^3	2.5×10^3
	Control		1.9×10^4		
Idem	1 hr	0, 0, 0	$>6.2 \times 10^4$(C)‡		$>9.5 \times 10^4$(C)
	5 "		4.5×10^4		6.8×10^4
	Control		6.2×10^4		
Isotonic virus (.14 M)	1 hr	0, 0, 1	$>1 \times 10^5$(C)	9.5×10^4	9.5×10^4
	Control		$>1.5 \times 10^5$(C)		
E_1 (.88 M)	1 hr	0, 0, 0	9×10^3	9×10^3	9×10^3
	3 "	0, 0, 0	4×10^3	2×10^3	5×10^3
	5 "	0, 0, 0	2×10^3	3.5×10^3	2.5×10^3
	Control		9×10^3		
Idem	1 hr	0, 0, 0, 0	2.5×10^2	1×10^3	1×10^3
	3 "	0, 0, 0, 0	1×10^3	1×10^3	1×10^3
	5 "	0, 0, 0	1.5×10^3	5×10^2	1.5×10^3
	Control		5×10^2		

* Plaque-producing particles/ml. † D_3T = .9 M with respect to NaCl. ‡ Confluent areas—count partly estimated.

of RNA as a result of different periods of time during which it was exposed first to distilled water, or subsequently to D_3T. Degree of infectivity recovered is not significantly different from degree found before dilution in distilled water.

Discussion. Loss of infectivity of RNA in distilled water, hypotonic or isotonic solutions and complete recovery of infectivity when the RNA is restored to a medium containing salt in hypertonic concentrations, has been shown to be a reproducible phenomenon. The significance of the phenomenon is clear only in that the reversibility of infectivity by hypertonic salt concentrations would seem to preclude participation of persistent RNAase in RNA preparations themselves as a major cause of inactivation in isotonic or hypotonic salt solutions. Inactivation of RNA infectivity by RNAase is not reversible.

TABLE III. Effect of Time Spent in Isotonic Solution on Titer of Ribonucleic Acid Further Diluted in Hypertonic Diluent at Intervals Noted.

	Time 10^{-1} dilution in D.W., hr	Titer on dilution in D_3T Stat	After 60 min.
Alcohol precipitated RNA from isotonic virus	1	1.1×10^5	1.4×10^5
	5	3×10^4	3×10^4
	18	5 "	9 "
	24	6 "	1.2×10^5
	Control	9 "	

Unexpressed infectivity of RNA may still be due to inactivation by RNAase of cells in test monolayers in low salt concentrations. Experiments to be reported later show that isotonic salt environments do enhance the effect of a host cell RNA inactivator, presumably RNAase. Preliminary observations suggest that RNA in low salt concentrations still does not produce infection despite reduction of the inactivator to undetectable levels by various means. The possibility that cell system inactivator plays an important part remains open, however. We have shown here only that RNAase plays no major role in inactivation of RNA in the diluent alone.

The theory proposed by Doty *et al.*(7) for the structure of RNA suggests that the molecule consists of a single strand of nucleoprotein with irregular hairpin-like helices along its length which straighten in iso- or hypotonic solutions and reform under hypertonic conditions. Infectivity responses to changes in molarity reported here may be the result of such changes in molecular structure. On the other hand, small changes in molarity which alter degree of infectivity can also be expected to change host cell surface permeability. Whether the salt acts on the host cell or on RNA itself, the data presented indicate that the RNA molecule in isotonic solutions is

still potentially biologically active; the expression of this activity is dependent on addition of salt.

Summary. In preparations of RNA made from polioviruses by phenol extraction technic, the infectivity of RNA which is rendered undetectable by dilution in isotonic or hypotonic salt solutions can be quantitatively recovered by addition of NaCl or KCl. This is true whether the exposure to low salt concentrations is brief, or as long as 24 hours. Reversibility of infectivity by hypertonic salt concentrations would seem to preclude participation of residual RNAase in the preparation itself as a major cause of inactivation of RNA infectivity with alterations in molarity.

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Effect of Vasopressin on Creatine Excretion in the Rat. (25500)

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In experiments designed to measure inhibition of postirradiation polydipsia and polyuria by antidiuretic hormone vasopressin, it was observed that 500 mU of Pitressin tannate in oil significantly increased urinary excretion of creatine in Co^{60} gamma irradiated rats as compared to irradiated non-injected controls(1). Evidence is presented here to show that a significant increase in urinary creatine excretion is also evoked in normal rats by injection of vasopressin.

Methods. Forty young female Wistar rats (135-282 g) were randomized by weight and injected intramuscularly with Pitressin tannate in peanut oil (Parke, Davis & Co.), aqueous Pitressin, commercial peanut oil (Planter's) or normal saline. Rats were housed in individual metabolism cages and fed Wayne Lab-Blox pellets except when noted; body weights, water intakes, and urine outputs were recorded daily. Twenty-four hour urine samples were analyzed for creatine by modification(1) of the method of Anderson *et al.*(2) and for creatinine by the Jaffe reaction after mechanically shaking (30 min) aliquots with 7 ml of Amberlite IRA-401 (30-60 mesh) in the chloride cycle (washed with 40 volumes of distilled water) and collecting the effluent by filtration over glass wool with 3 washes of distilled water. Recoveries of added creatinine averaged 90 $\pm$ 3%.

Results are summarized in Table I. In fed animals, significant creatinuria occurred on all 3 days after injection of 5 U Pitressin tannate in oil. Fasted control rats experienced the usual starvation creatinuria and Pitressin-evoked creatinuria was not significant until the second and third days. Of 5 rats injected with 20 U aqueous Pitressin, one died within 12 hours; in the remaining rats no significant increase in creatinuria was observed on either of 2 postinjection days. In experiment with fed rats, water intakes were significantly reduced on first postinjection day by Pitressin in oil ($P<0.01$) but no differences in water intakes were observed between fasted controls and fasted hormone-injected animals on first postinjection day. Water intakes of fed rats injected with aqueous Pitressin were not different from controls but urine outputs were significantly lower than controls on first postinjection day. In no experiment did body weights or creatinine excretions of hormone-injected rats differ from controls.