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Reaction of Infectious RNA with Mammalian Cells.* I. Attachment to Cells. (31655)

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In the course of studies to explain the marked discrepancy between the degree of infectivity in mammalian cells of whole polio virus and the infectious RNA derived from it, we noted(1) that infectious RNA apparently enters the host cell in at least two steps, 1) "attachment" which is independent of duration of exposure to monolayers, temperature, osmolarity and pH within the limits tested, and 2) "uptake" which is markedly influenced by these factors. Weil(2) using polyoma viral DNA and Borriss and Koch(3) using polio viral RNA, have reported similar findings. Experiments designed to study "attachment" of free polio virus RNA to mammalian cells in monolayers are described here.

The data to be reported suggest that the comparatively low titers of infectious RNA cannot be explained by inefficiency of attachment to cells. An average of 75% of the infectious units exposed to cell monolayers is removed by them in one minute. Previous investigators(4,5,6) who have reported poor attachment to cells used isotopically labelled cellular and viral RNA rather than infectivity as their quantitative tool.

Materials and methods. RNA—cell system.

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RNA was prepared by a previously described modification of the phenol technique(7) from Mahoney strain type 1 polio virus grown in HeLa cells. All RNA samples were used within 2-3 weeks of the time of preparation in order to avoid an unexplained titer increase which may be found in fluid drained from monolayers inoculated with RNA stored for more than a month in a dry ice chest. Monolayers consisted of HeLa (and occasionally amnion and HEP_2) cells grown in Eagle's medium to which 10% human serum and twice the usual concentration of vitamins and amino acids were added. The monolayers on 60 mm glass or plastic (Falcon) Petri dishes were washed twice with 2 ml isotonic solutions before inoculation as previously described. The second wash contained, where indicated, a final concentration of 1% of bentonite suspension prepared according to Singer and Fraenkel-Conrat(8). RNA inocula were prepared in solutions containing 10-15% bentonite suspension.

Use of bentonite. All RNA inocula exposed to cells for measurement of attachment were prepared in solutions containing approximately 10% bentonite. All such cells had been washed just before used with a solution containing 1% bentonite. Repeated experiments in our laboratory show that bentonite, under the conditions of the experiments to be described, protects RNA in salt concentrations as low as 0.14 M from higher concentrations of RNAase (0.01 μ/ml) than are found on monolayers(9).

Residual RNA. Polio RNA inoculum, 0.1 ml, was dropped on the center of the monolayers and allowed to spread over the surface. Three to six monolayers were used per test point. At varying intervals after inoculation plates were tilted and residual fluid containing the unattached or "residual" RNA was removed with a pipette (*i.e.*, "drained"). Since RNA inoculum adjusted to 1.3 M with respect to NaCl resulted in residual fluid which was found to be 0.56-0.6 M, the concentration of RNA in the residual fluid was assumed to be diluted approximately 1:1 by intracellular fluid and excess wash solution.

Attached RNA. Aliquots of residual fluid and original RNA inoculum appropriately diluted and adjusted to 1.3 M with respect to NaCl were tested for infectivity titer by seeding 0.1 ml quantities of appropriate dilutions on monolayers which were then incubated at 35°C for 20 minutes (or at room temperature for 30 minutes) before layering with agar. The difference between the number of plaque forming units demonstrable in the original inoculum and the number in the residual RNA sample provides a measure of plaque forming units removed and presumed, as a working hypothesis, to be attached to the cell surface.

RNA "uptake." RNA "uptake" by the cells was measured by allowing the original monolayers from which residual RNA had been drained to stand for a total of 30 minutes at room temperature before adding nutrient agar.

Results. Demonstration in preliminary experiments of rapid (within 1 minute) removal of the majority of available plaque forming units from preparations of polio virus RNA following exposure to cell monolayers suggested the existence of an attachment stage in infectivity as distinct from uptake and focussed attention on the factors which influence attachment. In brief, attachment is measured by exposing RNA to monolayers, removing the residual fluid from the monolayers and comparing the number of plaque forming units in the original inoculum with that in the residual fluid. The difference in titer between them provides a measure of the number of infectious units removed by exposure to cells, presumably by attachment.

Factors which influence attachment. Time. Fig. 1 shows that while the number of plaques produced by infectious RNA (upper graph) is markedly increased by increasing the duration of exposure to monolayers, there was no increase in attachment during the interval examined; the same number of plaque forming units of RNA were found in fluid removed from the monolayers after 1 minute and after 30 minutes exposure (lower graph). The data indicate that maximum attachment occurs in less than a minute.

Osmolarity. Fig. 2 shows the results of examination of the effect of osmolarity of the RNA diluent on attachment. While RNA

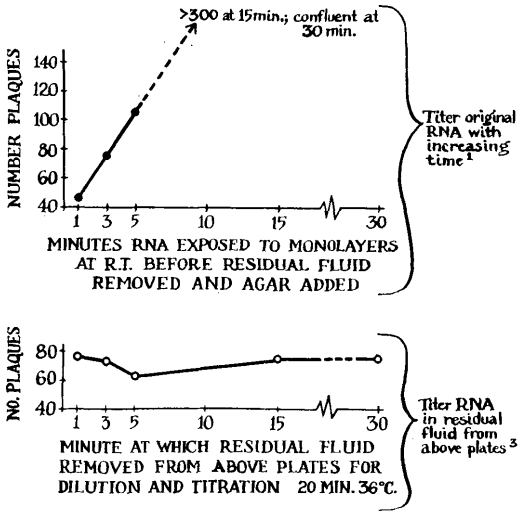


FIG. 1. Effect of duration of exposure of RNA to monolayers on titers of original RNA[1] and Residual Fluid[2] RNA[3]. 1. Titer of original RNA = measure of "uptake." 2. Residual Fluid (R.F.) = fluid removed from original monolayers at time noted. 3. Titer of R.F. — indirect measure of "attachment" (titer of "attached" RNA = titer of original RNA — titer R.F.)

infectivity clearly increases with increasing concentration of sucrose in the diluent (upper graph), comparable increases in osmolarity have no effect on "attachment" (lower graph). When the influence of changes in osmolarity of NaCl was examined, variation from 1.3 M to 0.5 M showed no effect on attachment of RNA to cells; at 0.3 M-0.4 M and below a reduction of 10-15% of plaque titer of residual RNA is found. Whether this change represents increased attachment or inactivation of infectivity of RNA is not known.

Temperature. Table I shows that attachment was not influenced by the temperature at which the experiments were carried out. The extremes which have been tested are 4°C and 37°C.

Similarly, pH, O_2 tension and antimetabolites were shown in other experiments to have no effect on attachment although their influence on infectivity was repeatedly demonstrated and is well known.

Proportion of seeded RNA which attaches to cells. If all the infectious particles which are exposed to monolayers and which cannot be drained from them after one minute are considered to be attached to the cells, then approximately 75% of the infectious units exposed to cells are removed by "attachment" (80%, 81%, 68%, 81%, 66%, 73%). The method for deriving the proportion is shown in the legend for Table I.

The average value of 75% attachment has been found under all conditions tested except when the salt concentration of the diluent is reduced to 0.3 M or less. In salt concentrations of 0.3 M or less, about 10-15% fewer infectious particles can be drained from monolayers.

Evidence that RNA removed from inoculum is attached to cells. Effect of washing on attachment (Fig. 3). The demonstration that RNA removed from the inoculum by exposure to cells can, for the first 20 minutes after exposure, be washed free from those cells and recovered quantitatively in the wash fluid offers strong support for the hypothesis that the RNA must have been attached (reversibly) to the cells rather than inactivated by some unknown mechanism.

Fig. 3 shows that washing reduces the plaque count on the washed monolayers by a quantity that decreases with time. Attachment of the average of 75% of infectious units exposed to cells is firm enough to withstand drainage (removal by gravity) of the residual fluid containing the unattached RNA, and, as noted, attachment occurs within a minute and does not vary within the time intervals tested. Consequently infectivity measured on drained monolayers (uptake) does not vary with time (●—● Fig. 3). However, flooding the plates or washing with 2 ml 0.6 M tris buffer NaCl‡ consistently reduces infectivity on such monolayers (O—O Fig. 3) by 60-70% at 5 minutes, 50% at 10 minutes, and by decreasing amounts up through 20 minutes.

Table II shows that the infectious units washed from the drained monolayers will form plaques in expected numbers on fresh monolayers. In the early attachment period (up to 20 minutes after inoculation) the second and third washes yield decreasing amounts of RNA. The sum of the infectious RNA titers

‡ 0.5-0.6 M NaCl is used as wash solution since this is found to be the NaCl concentration of the fluid which may be removed from monolayers after routine inoculation of RNA in 0.1 ml of 1.3 M NaCl.

TABLE I. Influence of Salt Concentration, Temperature, Exposure Time and Number of Infectious Units in the Inoculum on Number of Infectious Units in Residual RNA.

Attachment environment				(1)	(2)	(3)
Salt conc.	Temp.	Time	Dil.	Titr. original RNA, No. plaques/0.1 ml	Infectivity residual RNA drained from plates in column 1, No. plaques/0.1 ml at 2×10^{-3} dilution original RNA*	Calculated titer† residual RNA, No. plaques/0.1 ml
1.4 M	RT	15′	10^{-1}	Conflu	12	600‡
		30′	10^{-1}	"	15	750
		30′	10^{-2}	"	13	65
		30′	10^{-3}	58	—	
0.3 M	RT	15′	10^{-1}	20	20	1000
		30′	10^{-1}	52	16	800
		30′	10^{-2}	3	16	80
1.4 M	4°C	15′	10^{-1}	38	23	1250
		30′	10^{-1}	39	18	900
		30′	10^{-2}	6	14	70

* Salt concentration adjusted to 1.3 M NaCl. Inoculated plates incubated 20′ at 36°C.
† Titer calculated from data in column 2 and dilution factor.
‡ No. plaques column $2 \div 2 \times 100$ (residual RNA seeded 2×10^{-3} dilution of original RNA).

For purposes of illustration only (since original RNA and residual RNA titered at different times and temperatures) the proportion of RNA attached can be calculated from the data shown: 58×10^3 infectious units/0.1 ml were in original RNA exposed to cells. An average value of 8×10^3/0.1 ml infectious units remained in residual fluid (column 2 data $\div$ 2), $\frac{58-8}{58}$ units attached, or 87%.

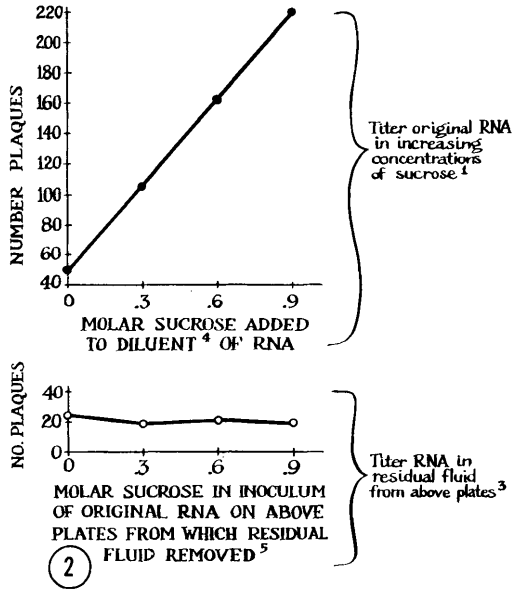
of residual fluid, repetitive wash fluids and on original plates is usually at least equal to and often greater than the titer of the RNA on control plates inoculated in routine fashion.

Use of cells in suspension. It is clear that the plaque forming units are removed from the RNA solution after exposure to monolayers and not inactivated. However the question whether the RNA removed is actually attached to cells remained to be answered. Since as large a proportion of available infectious particles was shown to attach to cell free glass or plastic Petri dishes as to monolayers, provided the test surface has been moistened with a saline solution containing at least 7×10^{-3} M NaCl, it was important to establish that the high proportion of attachment was to cells and not to cell-free surfaces. Consequently cells in suspension were examined for their capacity to remove RNA from solution. Duplicate samples of the same infectious RNA preparation in glass centrifuge tubes were diluted in 1.3 M and 0.14 M NaCl with bentonite added so that exposure to glass was as nearly identical as possible in all 4 tubes. To one tube of each pair a suspension of cells was added. To the other tube, cell diluent alone was added. The tubes were shaken frequently by hand for 30 minutes at which time cells

TABLE II. Quantitative Recovery of Infectious Units After Attachment to Monolayers.

	Total No. plaque forming units/ml in hypertonic NaCl				
	1	2	3	4	
Exp #	Original inoculum	Residual fluid	Wash fluid	Original plates (uptake)	Sum of p.f.u. columns 2-4 (compare col. 1)
1	50,000	12,100	9,700	30,000	51,800
2	176,000	48,000	34,000	>40,000	>135,000
		2nd wash	12,000		
		3rd wash	1,000		
3	20,000	10,000	4,000	8,800*	22,800

* Uptake on control plates not washed at 10 min = 17,100.



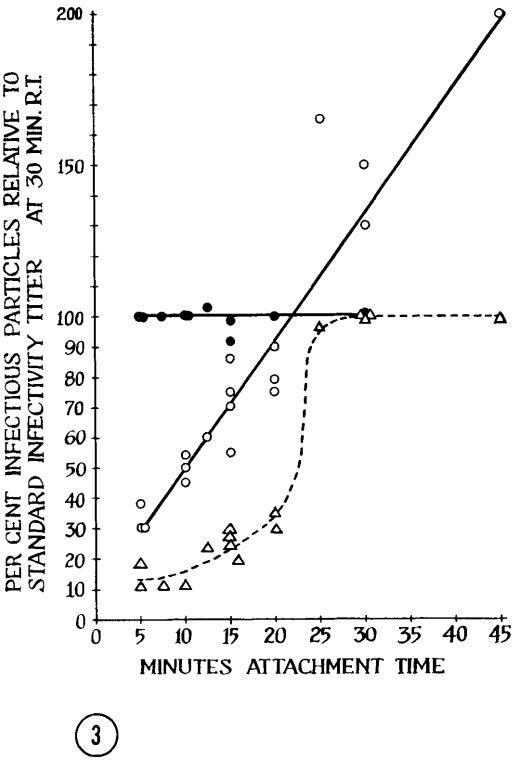


FIG. 2. Effect of sucrose concentration of diluent on titer of original RNA[1] and residual fluid[2] RNA[3]. 1. Titer or original RNA = measure of "uptake." 2. Residual fluid (R.F.) = fluid removed from monolayers after 30 min incubation time. 3. Titer of R.F. = indirect measure of "attachment" (see legend, Fig. 1). 4. Diluent = tris buffered 0.7 M NaCl & 1/6 bentonite. Incubated for 30 minutes at room temperature. 5. R.F. diluted, molarity adjusted to 1.3 M NaCl. Incubated for 20 min at 36°C.

FIG. 3. Uptake determined on drained monolayers and washed monolayers layered at time noted (composite of several experiments). ●—● Monolayers drained of surplus inoculum at time noted and left at room temperature to 30′. O—O Monolayers washed at time noted and left at room temperature to 30′ (after 30′ layered immediately). △ - - - △ Monolayers layered at time noted (standard infectivity titration).

were removed by centrifugation and the titers of the supernates compared. Table III shows data from an experiment demonstrating that addition of cells to RNA removes the majority of available infectious particles. Sixty-six per cent of the available infectious particles were removed by addition of cells to RNA diluted in 1.3 M NaCl and 71% by addition of cells to RNA in isotonic saline.

TABLE III. Removal of Infectious RNA Units from Supernate by Cells in Suspension.

RNA diluent	Avg No. p.f.u. in supernates in tubes to which Cell diluent added	Cells added	% p.f.u. removed by cells
1.3 M NaCl	63	21	66%
0.14 M NaCl	21	6	71%

Discussion. There is reason to believe that the rapid removal of infectious units of RNA from solutions exposed to cell monolayers is the result, in large part at least, of attachment to cells. The possibility that infectious RNA exposed to cells is inactivated in the process, possibly by RNAase in the cell environment, for example, is not substantiated by the experimental results. The quantity of RNAase which can be washed from monolayers does not decrease infectivity of RNA in hypertonic solutions nor does it decrease infectivity in isotonic solutions in the presence of the 1:10 dilution of bentonite used in all our experiments. The best evidence that the majority

of the infectious units removed are attached to cells is that appropriate washings of the monolayers within 5 minutes of inoculation can prevent plaque formation of 60-70% of infectious units seeded; the removed plaque forming units can be found in the wash solutions.

The evidence is conclusive that attachment is a stage in the entry of RNA into a host cell distinct from "uptake." The problem lies in quantitation of the proportion of RNA which is attached. The use of some RNA marker in addition to infectivity, such as an isotope label, would be desirable but has not proved helpful because the infectious fraction of labelled viral RNA makes up such a very small part of the whole. Holland *et al*(5) noted that while infectious RNA was removed from solution in quantity by exposure to cells, the proportion of the total P^{32} labelled polio RNA removed was too small to measure. More recently Borriss and Koch(3) demonstrated that the bulk of such labelled RNA is depolymerized and presented data indicating that a higher proportion (2-80%) attaches to cell surface when the highly polymerized RNA fraction is used. However, their results demonstrate an attachment rate of only 2-5% in the hypertonic solutions in which infectivity of RNA is at its height, and from 18-80% in isotonic solutions, a point which is difficult to explain. The most reliable tool now available for the study of infectious RNA seems to be infectivity itself. Using infectivity as the tool and accepting standard titration of the sample as a measure of total available infectious particles, our data show an average attachment rate of 75%. This is considerably higher than that reported by others(4,5,6) except for scattered values obtained by Borriss and Koch in isotonic solutions(3).

Norman and Veomett(6) and Borriss and Koch both noted an increase in attachment rate in isotonic solutions which is not explained. Borris and Koch used suspended cells which agglutinate heavily in high salt concentrations, thereby presumably reducing cell surface areas, but saturation of attachment sites was not demonstrated by them or by us. While we find some decrease in residual RNA in low salt concentrations, even if it is valid to interpret this result as increased attachment, the enhancement is only of the order of 10-15%. We consider RNA inactivation by a cell surface reaction (RNAase?) a more likely explanation under the circumstances of our experiments. While the bentonite used in our diluent neutralizes all measurable RNAase activity which can be washed from cell surfaces, this does not rule out some residual activity which might be present in isotonic environment on the cell surface.

The existence of attachment as a stage separate from uptake in the entry of nucleic acids into a cell has been noted before. Weil (2) commented that the "subviral infectious agent" of polyoma virus is rapidly attached to the cell membrane and that attachment is not affected by temperature and osmolarity which markedly influence uptake and subsequent infectivity. Borriss and Koch published more fully on "adsorption"(3), a process we label as "attachment" to distinguish it from whole virus adsorption. Our findings differed from theirs in two respects: 1) We found no evidence in our experiments for their interpretation that RNA not "adsorbed" in the first exposure to cells is poorly "adsorbed" in the second, and 2) They found that maximum attachment required 20-22 minutes and was irreversible. However, attachment was irreversible in both laboratories after 20-22 minutes, and their suspended cells test system did not lend itself to detection of the reversible early attachment observed in ours.

Summary. Infectious polio virus RNA enters mammalian cells in tissue culture in a 2-step process: 1) "attachment" which is independent of time, temperature, osmolarity, pH or other factors in the ranges known to influence infectivity of RNA, and 2) "uptake" which is markedly influenced by these factors. An average of 75% of plaque producing RNA units exposed to cells in monolayers becomes attached to them in less than 1 minute.

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Effect of Chronic Lead Intoxication on *in vivo* I^{131} Uptake By the Rat Thyroid.* (31656)

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Lead poisoning continues to be an important industrial hazard, and in recent years has become a problem affecting persons who consume illicit whiskey(1). We have found impaired I^{131} uptakes responsive to TSH in patients with plumbism and have suggested that this abnormality is caused by the metal (2). Porritt(3) previously had suggested an impairment of thyroid function by lead in 1931, and Kremer and Martin(4) reported a case of coexisting myxedema and plumbism in 1955.

To test this hypothesis, rats were chronically intoxicated with lead and *in vivo* radioactive iodine uptakes measured. The findings are the subject of this report.

Methods. Male and female weanling Sprague-Dawley rats were placed in cages, 3 of a sex to a cage, and fed Purina laboratory chow. Tap water was given the control animals; lead acetate dissolved in tap water in concentrations of 1 g/liter and 4 g/liter was given the experimental group. After 6 months all animals were fed a Remington low-iodine diet for 2 months and 24-hour *in vivo* I^{131} uptakes were determined. The uptakes of 6 female and 3 male controls were essentially the same as 7 females and 6 males receiving 4 g of lead acetate/liter of drinking water. The concentration of lead acetate was then increased to 8 and 12 g/liter of water. These concentrations of lead (1, 4, 8, 12 g/liter) were given because previous studies had shown that two effects of lead intoxication, anemia and renal tubular inclusions, occur in the rat at this dose level, without death of the animal. Thyroid studies were repeated after 2 to 6 months of feeding Purina laboratory chow plus the increased ingestion of lead. The low-iodine diet was fed for 2 weeks and the *in vivo* thyroid I^{131} uptake determined at 1, 2, 3, 6, 12, and 24 hours. The uptake curve was determined because previous measurement of the uptake at 24 hours had shown little difference between the experimental and control animals. The rats were killed at the end of the expirement and the conversion ratio, *in vitro* I^{131} uptake, thyroid weight, and hematocrits were measured. Urinary and fecal excretion of I^{131} was not measured.

In vivo I^{131} uptake was determined using light nembutal anesthesia and subcutaneous administration of sufficient I^{131} to give epithyroid counts at 1 hour of at least 9 to 10 times background. The rats were tied to a wooden stand over which a 1/2 inch thick lead shield, having a 1/4 inch diameter hole, was placed. The shield was flush with ventral surface of the rat with the hole directly over the thyroid. A wide field columator with sodium iodide crystal was placed directly above the hole in the shield. Emissions were counted using a Nuclear of Chicago model 132B coun-

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