ENTEROVIRAL RIBONUCLEIC ACID

I. Recovery from Virus and Assimilation by Cells*

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Recognition of the infectivity of ribonucleic acid (RNA) of tobacco mosaic (1, 2) and of animal virus (3) initiated investigation of the molecular aspects of viral reproduction. Study of replication of infectious RNA depends upon employment of efficient and reproducible biological assay procedures. Reception of viral genetic material normally depends upon specific interaction of cellular and viral receptors (4–6) while phenol extracted virus has lost this specificity (7). Therefore, assay of infectious RNA may require extracellular environments different from those suitable for assay of whole virus.

Plaque formation of infectious RNA was greatly improved by use of 1 M sodium chloride solution as described by Alexander *et al.* (8), but considerable variation among enteroviral RNA titers was noted when different RNA preparations were tested in a number of established human cell strains. The general procedure of Gierer and Schramm (1) consistently yielded infectious poliovirus RNA and minor alterations in the extraction procedure were ineffective. Much of the inefficiency of viral RNA action apparently results from failure to introduce RNA into replicating sites of cells. This paper reports factors affecting the biological activity of enteroviral RNA, and a plaque assay system that has proved sensitive and reproducible. Production of infectious RNA from highly purified poliovirus is reported, in confirmation of the observations of others (9, 10), and evidence is presented that only a small fraction of total extracted virus RNA is adsorbed to cells, and is responsible for infectivity.

Material and Methods

Cell Cultures and Methods.—Methods and media used for routine cultivation of HeLa cells and monkey kidney, human amnion, and rabbit skin fibroblast cells in primary culture have

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been described previously (5). L strain mouse fibroblast cells were propagated from the strain maintained by Scherer (11).

Viruses.—Type 1 poliovirus (Mahoney) was employed as pooled fluid of the 9th and 10th HeLa culture passage of virus received from Connaught Medical Laboratories, Toronto; type 2 poliovirus (MEF-1) was grown in monkey kidney cells or established human cell lines after reception from Connaught Laboratories; Coxsackie B1 (Conn. 5) virus was purchased from The American Type Culture Collection and used after 4 to 5 HeLa passages.

Purified viruses were prepared by ultracentrifugation and column chromatography on diethylaminoethyl cellulose and, where indicated, were labeled with radiophosphorus as described by Hoyer *et al.* (12, 13).

RNA Preparation.—Virus for extraction was suspended in 0.02 M phosphate buffer at pH 7.2, supplemented with 5×10^{-4} M ethylenediamine sodium tetraacetate (EDTA) and 10^{-2} M tris(hydroxymethyl)amino methane (tris) or veronal buffer at pH 7.2. RNA was extracted with phenol at room temperature (1), and stored at 0°C. until used.

RNA Infectivity Assay.—The following procedure was found optimal for sensitive and reproducible assay of viral RNA infectivity. Assay cultures of HeLa cells dispersed from donor cultures grown in human serum medium were seeded lightly in medium containing 20 per cent calf serum in YEM (5). After 2 to 4 days, the cells multiplied to form nearly confluent, firmly adherent monolayers of about 2×10^6 cells. It should be emphasized that this exposure of HeLa and other cells to calf serum following production in human serum medium was found necessary, as it increased cell adherence to glass and reduced susceptibility to hypertonic shock, compared to similar exposure of cells to human, horse, or rabbit serum. The RNA solution was diluted 1:10 into 2.2 M magnesium sulfate solution (further dilution was made in the 2 M magnesium sulfate solution). To minimize possible loss of infectivity by aggregation, RNA solutions were diluted into magnesium sulfate solution just before addition to monolayers; highly concentrated RNA solutions, which would quickly aggregate in $MgSO_4$, were first diluted in 10^{-2} M tris, pH 7.2. Monolayers, rinsed 3 times with Hanks' balanced salt solution, were inoculated at room temperature with 0.2 ml. of RNA in 2 M $MgSO_4$, incubated for 12 to 15 minutes with frequent rocking, washed twice with BSS, and overlaid with 0.6 per cent Difco agar medium containing 20 per cent calf serum and 0.1 per cent yeast extract in BSS. After 2 to 3 days at 37°C., the overlayer was removed and the cells were stained with crystal violet for enumeration of plaques (14).

Virus Assay.—Assays for poliovirus and Coxsackie virus were made by plaque count as described previously (14).

Enzymes.—Crystalline-ribonuclease (Nutritional Biochemicals Corp., Cleveland) treatment of extracted viral RNA was employed as a routine control measure. Infectious RNA preparations described were inactivated by this treatment.

EXPERIMENTAL

Physical and Chemical Factors Affecting Assay of RNA Infectivity.— The standard method for assay of RNA infectivity described in Materials and Methods was developed through analysis of effects of temperature, time of cell exposure, and constituents of the menstruum. Table I shows effects of some of these factors in comparison with the standard procedure. RNA infectivity of cells exposed to RNA in $MgSO_4$ was little affected by pH changes from 6 to 8 but was inhibited by low temperature. Elevation of the temperature of exposure to 37°C. produced no apparent increase in the number of plaques produced per unit inoculum of RNA, but visibly increased the extent of mor-

phologic distortion of cells exposed to hypertonic salt solution. In hypertonic concentration, chloride anions (as magnesium chloride) were more destructive to cells than were sulfate ions. Mixed sodium chloride and magnesium sulfate allowed efficient RNA infection, although not as good as 2 M magnesium sulfate alone. Table I also shows that the method of Alexander *et al.* (8) and its later modification (15) were less sensitive for RNA assay with the cultures of HeLa cells employed here, even in the absence of a chelating agent (EDTA reduces

TABLE I

Relative Infectivity for HeLa Cells of RNA Extracted from Type 1 Poliovirus by Various Methods

Procedural variation	RNA infectivity (per cent of maximum titer)
None (standard method)*	100
1 M NaCl (Alexander *et al.* (8))	0.1–20
0.9 KCl, pH 8.0 (Sprunt *et al.* (15))	0.1–10
Standard method at 37° C	100(CD)‡
Standard method, 5 min. exposure	30–60
Standard method, 30 min. exposure	10–35(CD)‡
2 M NaCl pH 7.2 (tris)	−(SCD)§
2 M $MgCl_2$ pH 7.2 (tris)	−(SCD)§
1 M NaCl, 0.5 M $MgSO_4$ (total 1.5 M) pH 7.2 (tris)	10–60
2 M $CaCl_2$ pH 7.2 (tris)	−(SCD)§
0.2 M $MgSO_4$ pH 7.2 (tris)	<1.0
2 M sucrose pH 7.2 (tris)	<0.01
Standard method pH 6.0	100 (>90)
Standard method pH 8.0	100 (>90)

* Rinsed HeLa monolayers exposed 12 to 15 minutes at room temperature to 0.2 ml. RNA in 10^{-2} M tris-buffered 2.0 M $MgSO_4$, rinsed 2 times with BSS, and overlaid with 20 per cent calf serum in BSS containing 0.1 per cent yeast extract (medium solidified with 0.6 per cent Difco agar).

‡ CD, cell damage.

§ SCD, severe cell damage (plaque counting not possible).

the efficiency of RNA assay by the method of Alexander *et al.* (8) presumably by chelation of residual divalent cations). The enhancing effects of high pH obtained with the monovalent cation assay (10) were not observed using $MgSO_4$.

By use of HeLa cell monolayers maintained in calf serum medium, and the relatively non-damaging 2 M magnesium sulfate solution, it was possible to obtain more efficient assay of infectivity of RNA from type 1 poliovirus and Coxsackie B1 virus (Table II), and satisfactorily reproducible assay (Table III). Plaque-forming units from RNA were consistently 0.1 per cent or more of those produced by the whole virus from which it was extracted.

Evaluation of the dose response relation for HeLa cells exposed to poliovirus

RNA revealed a linear regression through the origin to suggest that a single element could be infective (Fig. 1). This observation is in agreement with the findings of Alexander *et al.* using 1 M NaCl (8).

Fig. 2 shows the effect of varying concentrations of magnesium sulfate solu-

TABLE II

Infectivity of Whole Virus and Extracted Ribonucleic Acid (RNA)

Experiment No.	Whole virus titer	RNA titer	Per cent of whole virus infectivity
	Type 1 poliovirus		
1	2×10^{9}*	6×10^{6}	0.3
2	8×10^{9}	9×10^{6}	0.1
3	7×10^{8}	2×10^{4}	0.3
4	2×10^{9}	4×10^{6}	0.2
	Coxsackie B1		
1	8×10^{8}	7×10^{5}	0.1
2	2×10^{9}	3×10^{6}	0.1
3	5×10^{6}	9×10^{3}	0.2

* Plaque-forming units per ml. of original suspension, determined by inoculation of HeLa monolayers with virus or RNA.

TABLE III

Replicate Assay of RNA from Poliovirus and Coxsackie Virus

Experiment No.	Plaques per monolayer*
	Type 1 poliovirus
1	189, 157, 175, 191, 198, 219, 202
2	125, 131, 145, 150, 155, 132, 146, 119, 106, 140
3	123, 137, 140, 164, 136, 144, 172
	Coxsackie B1
4	83, 64, 77, 47, 113, 80, 75
5	238, 213, 232, 207, 217, 254, 225

* HeLa cell monolayers were rinsed and exposed to 0.2 ml. RNA diluted 1:1000 in 2.0 M $MgSO_4$ buffered with 10^{-2} M tris(hydroxymethyl)aminomethane, pH 7.2, for 12 to 15 minutes before washing and overlayering with agar medium.

tion as suspending menstruum on infectivity of poliovirus RNA for HeLa cell cultures. Since cells were morphologically altered by exposure to 0.2 ml. of 2 M magnesium sulfate solution, and were completely destroyed by exposure to large volumes of the solution, the enhancing effect of increasing salt concentration was presumably compensated by increasing cellular damage as the salt solution approached saturation.

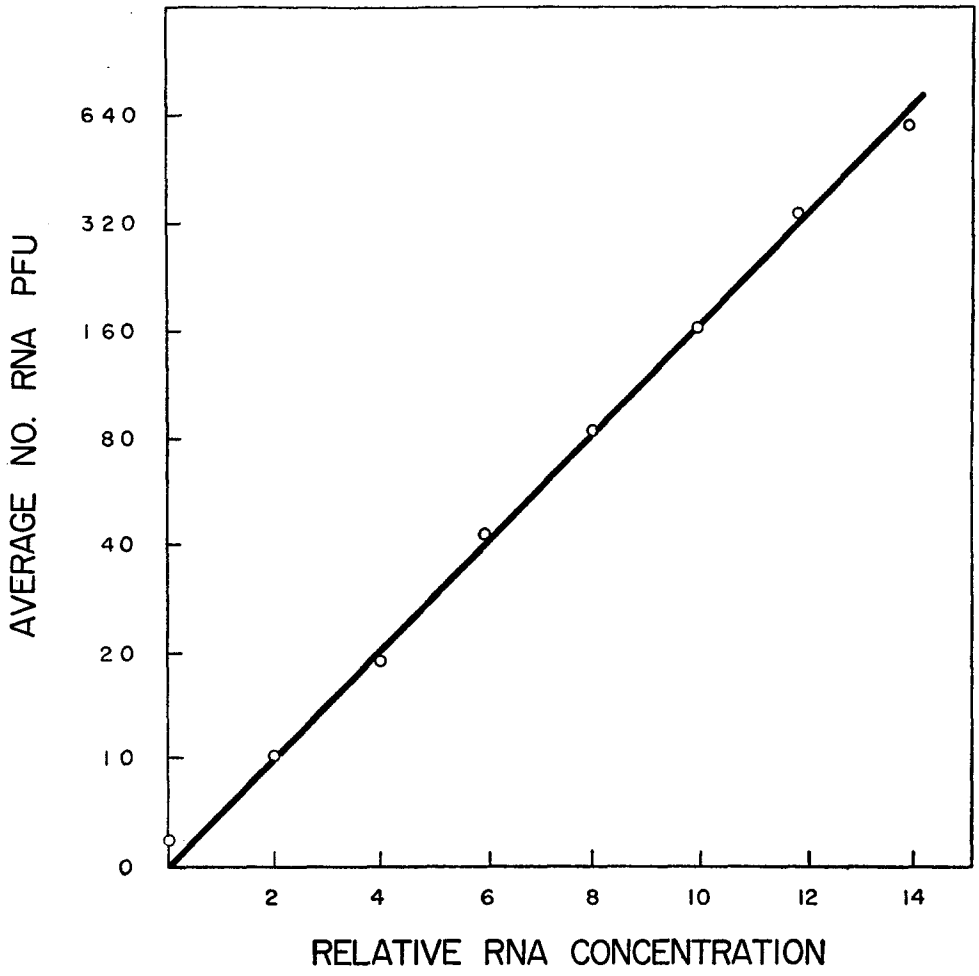

FIG. 1. Dose-response relation for type 1 poliovirus RNA extracted with phenol and placed on HeLa cell cultures in 2 M magnesium sulfate solution. Each point represents the average of 4 counts per twofold dilution.

Comparative Effects of Osmolarity on Cell Strains.—We have found that the optimal osmolarity of magnesium sulfate solution for exposure of cells to RNA differs among cells. Optimal salt concentration depends also on the conditions of cultivation, and therefore must be adjusted for each cell line and each cultural method employed. Under similar conditions of cultivation, other established human cell strains have shown a sensitivity to RNA infection about equal to that of our HeLa cells. Monkey kidney cells in primary cultures have proved less sensitive, and human amnion cells in culture are often refractory, under

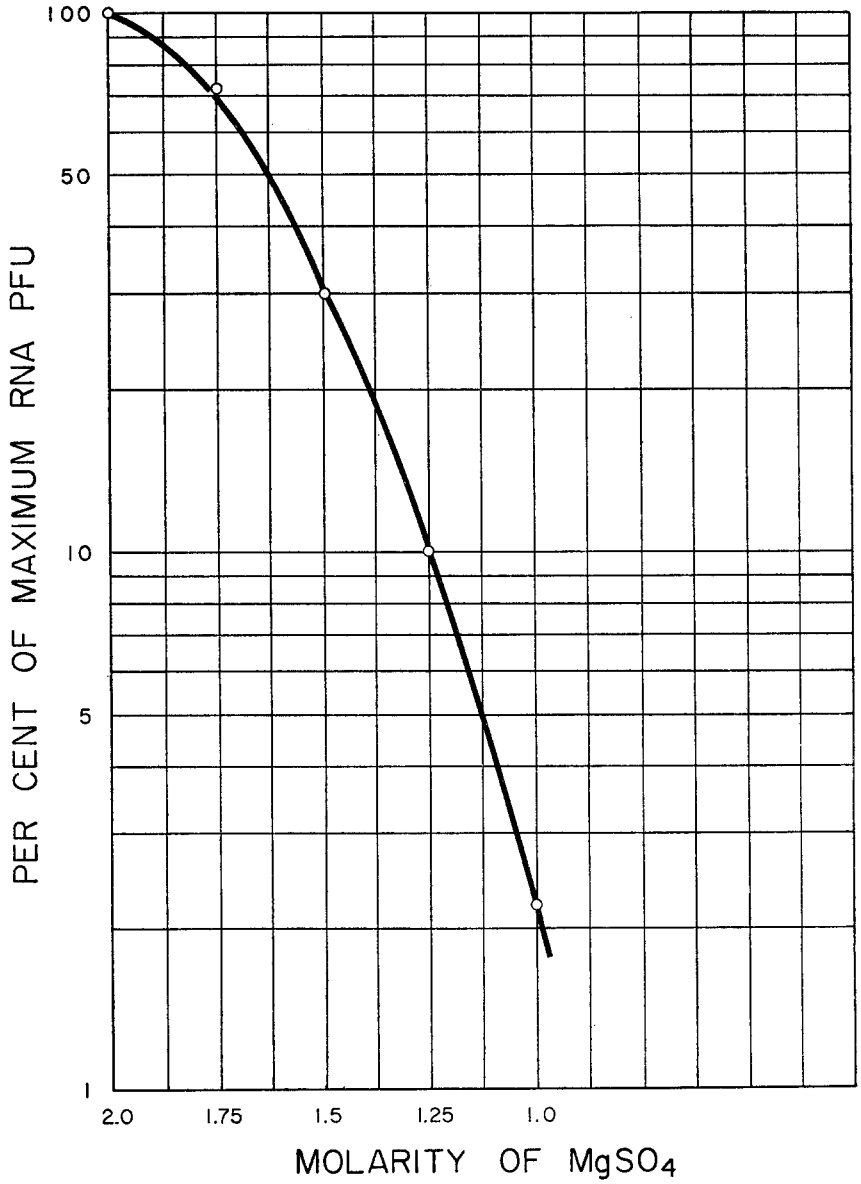

FIG. 2. Effect of increasing concentrations of magnesium sulfate solution at pH 7.2 as suspending medium for type 1 poliovirus RNA inoculated into HeLa cell cultures.

the conditions of our standard method. Naturally insusceptible L mouse fibroblasts were at least as susceptible to poliovirus RNA as HeLa cells, when L cell monolayers grown in calf serum medium were exposed to RNA in 2 M magnesium sulfate and harvested after 1 cycle of infection. In an exemplary

experiment, assay of total produced plaque-forming units and number of infected cells in exposed cultures showed 230 plaque-forming units per infectious center for HeLa cells and 400 plaque-forming units per infectious center for L strain cells with approximately equal number of infectious centers per cell. Primary rabbit skin fibroblasts, unlike HeLa cells, were destroyed by exposure to 0.2 ml. of 2 M magnesium sulfate solution, but underwent 1 cycle of infection and produced intact virus on exposure to poliovirus RNA contained in 1 M magnesium sulfate.

Efficiency of RNA Extraction.—Infectivity associated with extracted RNA represented less than 1 per cent of original intact virus, and possible reasons for this poor infectivity were sought. For investigation of the efficiency of the

TABLE IV

Recovery of P^{32}-Labeled RNA From Poliovirus Extracted with Phenol

P^{32}-labeled material	Per cent of P^{32} label remaining per ml.*		Per cent of RNA P^{32} acid-precipitable	
	Experiment 1	Experiment 2	Experiment 1‡	Experiment 2§
Original purified virus	100	100	—	—
RNA from the same virus	96	86	97	99

* Per cent of P^{32} label recovered following extraction of virus with phenol.

‡ Per cent of RNA P^{32} label precipitated by cold 25 per cent perchloric acid containing 0.7 per cent uranium acetate, and 5 mg./ml. yeast RNA as carrier.

§ Per cent of RNA P^{32} label precipitated by cold 95 per cent ethanol containing 1 N HCl and 5 mg./ml. yeast RNA as carrier.

extraction procedure, type 2 poliovirus was labeled with radiophosphorus and purified as described by Hoyer *et al.* (13), and phenol-extracted to release the RNA. Table IV shows that nearly all of the radioactive label remained in the aqueous phase after phenol and ether extraction, and that most of the label in the extracted RNA was acid-precipitable. From these results it appeared that most of the P^{32} in the purified virus was incorporated in the RNA, and that the relatively low infectivity of enteroviral RNA preparations could not be attributed to loss of RNA during extraction. Fig. 3 shows the ultraviolet absorption spectra of intact poliovirus and the extracted labeled RNA in 0.02 M phosphate buffer, pH 7.2, plus 5×10^{-4} M EDTA (the RNA curve is corrected for per cent P^{32} recovery). The large absorption increment at 260 mμ (27 per cent) obtained upon depolymerization of RNA with ribonuclease indicated that most of the absorbing material was highly polymerized. According to Hotchkiss (16), highly polymerized RNA preparations show an absorption increment up to 33 per cent when depolymerized. From Fig. 3, it is seen that the intact type 2 poliovirus absorbed ultraviolet light maximally at 260 mμ, and the RNA at 259 mμ; most of the absorption by virus at 260 mμ could be attributed to

the RNA which in its free state had a greater absorbance than the whole virus from which it was derived. Intact virus showed an absorption minimum at 241.5 mμ; the viral nucleic acid was sharply differentiated by its minimum at 229.5 mμ. The ratio of absorbance at 260 and 280 mμ was 1.70 for the intact

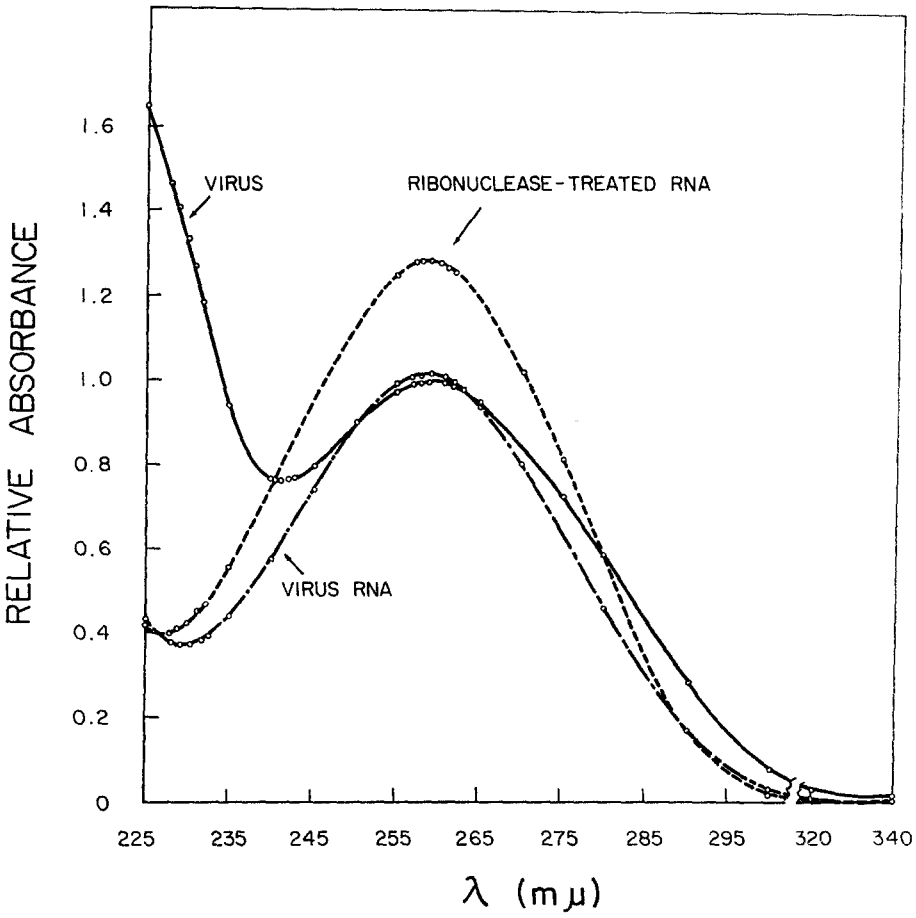

FIG. 3. Ultraviolet absorption spectra for chromatographically purified type 2 poliovirus, viral RNA extracted by the phenol method, and ribonuclease-treated RNA.

purified virus, and 2.17 for the RNA extracted from the same virus preparation. These results, together with the data on ultracentrifugation given hereafter, indicate considerable purity of the virus and its extracted RNA. The comparatively low infectivity of RNA compared to intact virus thus did not appear attributable to extensive loss of, or depolymerization of, RNA during phenol extraction.

It can also be seen in Fig. 3 that extracted viral RNA in 0.02 M phosphate buffer is less hypochromic than the RNA in the intact virus. This indicates that the RNA within intact poliovirus is at least partially hydrogen-bonded.

Cellular Reception of Poliovirus RNA.—We next correlated the cellular uptake of total virus RNA (radiolabeled physical particles) with the uptake of infectivity of virus RNA.

The process of cellular reception of poliovirus RNA infectivity was studied by addition of RNA in different ionic media to HeLa cell monolayers. After 15 minutes for adsorption, supernatant fluid was removed from the exposed cultures and assayed on other monolayers to detect residual unattached in-

TABLE V

Adsorption of Type 1 Poliovirus RNA Infectivity by Cell Monolayers

Treatment of RNA:	Residual RNA PFU after 15 min.	
	Experiment 1	Experiment 2
None (control RNA)	178, 180, 185	200, 186, 196
Adsorbed to HeLa cells in 2 M $MgSO_4$*	7, 3, 2	7, 7, 4
Diluted into 2 M $MgSO_4$ previously placed over HeLa cells‡	182, 177, 166	205, 208, 178
Adsorbed to L strain mouse cells in 0.1 M $MgSO_4$	0, 0, 0	2, 0, 0
Adsorbed to HeLa cells in 0.1 M $MgSO_4$	2, 1, 3	1, 1, 2

* Type 1 poliovirus RNA containing about 10^4 PFU was added to HeLa cell monolayers (2×10^6 cells) in 0.2 ml. of the indicated salt solution. After 15 minutes of rocking for adsorption, the supernatant fluid was carefully removed, diluted 1/10 in 2 M $MgSO_4$ and assayed for residual unattached RNA plaque-forming units (PFU).

‡ 2 M $MgSO_4$ had previously been placed over HeLa cell monolayers for 15 minutes in 0.2 ml. amounts per 2×10^6 cells.

fective units. Representative results are shown in Table V. The infectivity of poliovirus RNA was taken up rapidly by HeLa cells whether added in higher or lower ionic strength menstruum. Thus, the effect of hypertonic salt solution was not to increase adsorption.

In contrast, when adsorption of P^{32}-labeled poliovirus RNA was studied it was found that the bulk of RNA remained in the menstruum; no detectable adsorption of P^{32} RNA from the medium was observed (Table VI). Thus, it can be concluded that the major loss of infectivity accompanying extraction of RNA from whole virus resulted because only a very small proportion of the RNA (not detectable within the limits of radioassay) was taken up by cells, although this small infectious fraction was received rapidly. In the previous experiment (Table VI) adsorption times were kept to a minimum to avoid possible complications due to cellular uptake, followed by hydrolysis, and release of hydrolytic products of P^{32} RNA.

It was desirable to verify that uptake of only a small fraction of RNA was the reason for the low infectivity, and that cellular reception of infectivity was being measured rather than enzymic hydrolysis or other inhibition of RNA by materials released from HeLa cells during exposure to 2 M magnesium sulfate solution. Many HeLa cell monolayers were exposed to 2 M magnesium sulfate solution for 15 minutes (0.2 ml./10^6 cells); this solution then was removed and pooled, and used as suspending menstruum for poliovirus RNA. As seen in Table V, incubation for 15 minutes in this magnesium sulfate solution did not inactivate RNA infectivity. Thus, HeLa cells exposed to 2 M $MgSO_4$ did not release materials inhibitory for infectious RNA, and the previous experiment

TABLE VI

Adsorption of P^{32}-Labeled Type 2 Poliovirus RNA to HeLa Cell Monolayers under Conditions Which Yield Maximum Infectivity

Time of adsorption to HeLa cells*	Counts per min. recovered from supernatant fluid		
	Adsorbed in		Per cent P^{32} RNA adsorbed
	2 M $MgSO_4$	1 M NaCl	
0 (control)	141	154	—
5 min.	154	164	<10
10 min.	138	170	<10

* P^{32}-labeled type 2 poliovirus was extracted with phenol to obtain P^{32}-labeled RNA. This RNA was diluted into either 2 M $MgSO_4$ or 1 M NaCl, and placed in 0.2 ml. amounts on washed, thoroughly drained HeLa cell monolayers. The bottles were rotated at intervals to distribute the inoculum. Supernatant fluids were carefully removed after indicated periods and residual unadsorbed RNA determined by radioactivity counts.

was most likely measuring cellular uptake of RNA, rather than extracellular degradation.

HeLa cells exposed for 15 minutes to poliovirus RNA in 2 M magnesium sulfate solution, then washed and treated with ribonuclease, yielded as many plaques as untreated RNA-exposed cells. These results suggested that the infectious fraction of RNA attached to cells and penetrated rapidly. That this attachment was not due to specific interaction between cell receptors and residual virus surface protein is shown (Table V) by the equally rapid adsorption of RNA infectivity to L strain mouse fibroblasts which lack receptor for intact poliovirus (6).

Role of Hypertonic Salt Solution in Infection of HeLa Cells by Poliovirus RNA.—Since the infectious fraction of extracted poliovirus RNA was received by HeLa cells equally well in high or lower ionic strength solution, it was possible that the primary effect of hypertonicity was to promote cellular activity; *e.g.*, pinocytosis of adsorbed RNA. To investigate this possibility, HeLa

cell monolayers were exposed to poliovirus RNA in low ionic strength magnesium sulfate solution, then washed and overlaid for plaque counts, or washed and exposed to 2 M $MgSO_4$ to promote infection by adsorbed virus before overlaying. Although the sequential exposure in comparison with exposures to only low ionic strength medium increased the efficiency of infection more than tenfold (Table VII), the yield of infectivity was small compared to that found with cells exposed by the standard procedure. This recovery of some of the RNA infectivity lost to cells during exposure in low ionic strength medium suggests that at least a portion of the RNA was attached to, or within, cells, and was not degraded. But the relatively poor recovery as compared to the standard method also suggests that high salt concentrations enhance RNA infectivity

TABLE VII

Response to Type 1 Poliovirus RNA of HeLa Cell Monolayers Exposed to Hypertonic Salt Solutions during and after Adsorption

RNA adsorption time and medium*	RNA titer‡	Maximum RNA PFU recovered
		per cent
10 min., 2.5 M $MgSO_4$ (control)	8×10^4	100
10 min., 0.2 M $MgSO_4$	2×10^2	0.25
10 min. in 0.2 M $MgSO_4$, then cells washed with 0.2 M $MgSO_4$, then with 0.2 ml., 2 M $MgSO_4$	7×10^3	9

* Following adsorption of 0.2 ml. of virus RNA and washings as indicated each HeLa cell monolayer was further washed with BSS and overlayed for plaque development and counting.

‡ Titers are averaged from plaque counts obtained from 3 monolayers at each dilution of RNA tested.

not only by increasing cellular incorporation of adsorbed RNA, but also by maintenance of the RNA in a more infectious state during adsorption (since RNA infectivity lost during cell exposure to 0.2 M magnesium sulfate solution was not completely restored by subsequent application of 2 M magnesium sulfate solution). Conceivably, the promoting effect of 2 M magnesium sulfate on poliovirus RNA infectivity could result from induction of extensive hydrogen bonding and tight coiling with a single RNA chain. A tightly coiled chain might occupy a smaller area upon attachment to the cell surface, possibly facilitating entrance into the cell. RNA molecules with extensive hydrogen bonding would also be less sensitive to cleavage by hydrolytic enzymes (17).

Multiple functions of high salt concentration on poliovirus RNA infectivity for HeLa cells were indicated further by the influence of hypertonic sucrose solution in presence and absence of low ionic strength concentrations of magnesium sulfate solution. Table VIII shows that less than 1 unit of a thousand infectious units of poliovirus RNA could generate a plaque when added to

HeLa cell monolayers in 2 M sucrose solution alone or 0.1 M magnesium sulfate solution alone, while a significantly greater number of plaques were produced by infectious units suspended in a mixture of the two solutions. When some divalent cations were present, the hypertonic non-ionizing solution promoted infectivity of viral RNA, implicating hypertonicity itself.

Despite the promoting effect of divalent cations on infection of cells by viral

TABLE VIII

Effect of Hypertonic Sucrose on RNA Infectivity in the Presence and Absence of Divalent Cations during Exposure to Cells for Plaque Assay of RNA Infectivity

Virus RNA adsorbed to HeLa cells for 15 min. in:	RNA titer*	Maximum RNA infectivity
		per cent
2.0 M $MgSO_4$ (control)	2×10^4	100
0.1 M $MgSO_4$ only	$<10^1$	<0.1
2.0 M sucrose only	$<10^1$	<0.1
2.0 M sucrose containing 0.1 M $MgSO_4$	2×10^2	1

* Following 15 minutes adsorption of type 1 poliovirus RNA in 0.2 ml. of the indicated medium the HeLa cell monolayers were washed with BSS and overlayed with agar medium for plaque development. Titers are averages of 3 determinations.

TABLE IX

Lack of Effect of Strong Chelation on Maintenance of Infectivity of Poliovirus RNA

Treatment of RNA	RNA titer
None (control)	3×10^6
1 M citrate*	3×10^6
1 M EDTA*	4×10^6

* Poliovirus RNA was treated for 10 minutes at 25°C. with the indicated chelating agent, then diluted 10^{-3} in distilled water before final dilution into 2 M $MgSO_4$ for assay of infectivity.

RNA it appears that divalent cations are not necessary for maintenance of infectivity of RNA, for RNA can be stored at 0°C. in 5×10^{-4} M EDTA for over 1 week with no detectable loss of activity. Furthermore, treatment of infectious RNA with very high concentrations of EDTA or citrate failed to reduce infectivity if the treated RNA was later diluted and exposed to cells in 2.0 M $MgSO_4$ (Table IX). Thus, if multivalent cations bound to RNA are essential for maintenance of infectivity, they must be bound covalently. It has recently been shown that nucleic acids from a variety of sources do contain high concentrations of firmly bound metals which are not removed by chelating agents (18). Fraenkel-Conrat (19) has shown that low concentrations of EDTA promote stability of tobacco mosaic virus RNA, and a 0.1 M concentration of EDTA only slightly reduced infectivity.

Homogeneity of Poliovirus RNA Extracted by the Phenol Method.—The rapid adsorption of infectivity of viral RNA by cells, and cellular failure to adsorb a significant proportion of total extracted RNA measured by the radioactive label, suggested that infectivity either resided in a fraction differing from the

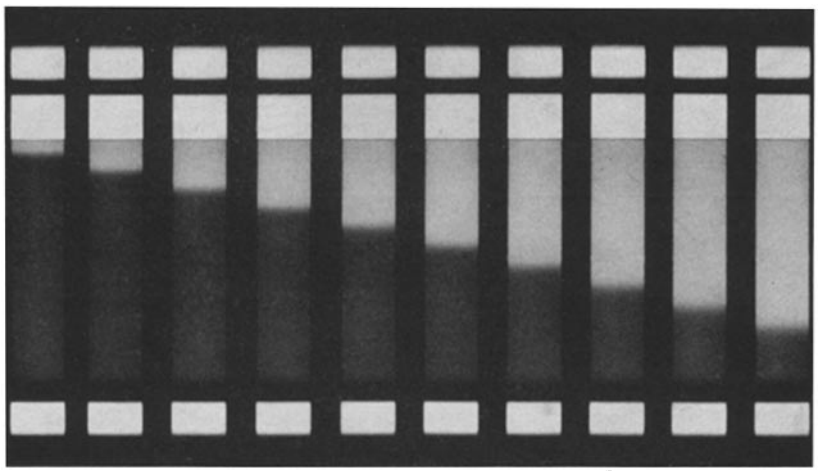

FIG. 4. Boundary of chromatographically purified intact type 2 poliovirus sedimented at 15,220 R.P.M. at 10°C. in the analytical ultracentrifuge, as revealed by ultraviolet optics at 8-minute intervals. $s_{20,w} = 147 \pm 4S$. Virus concentration was 0.7 mg. (1.4 O.D. units) per ml. of 0.1 M NaCl solution.

FIG. 5. Pattern of poliovirus RNA sedimented at 50,740 R.P.M. at 10°C. in the analytical ultracentrifuge, as seen at 8-minute intervals. RNA was prepared from virus with sedimentation pattern shown in Fig. 4. RNA concentration was 0.4 mg. (2 O.D. units) per ml. of 0.1 M NaCl solution containing 0.02 M phosphate buffer (pH 7.4) and 5×10^{-4} M EDTA. $s_{20,w} = 37 \pm 2S$ for the fast moving boundary.

bulk of the extracted RNA, or that efficiency of adsorption and reception by cells was poor owing to rapid uptake, hydrolysis, and release by cells. Fig. 4 shows the sedimentation pattern of intact type 2 poliovirus in the analytical ultracentrifuge, as revealed by ultraviolet optics. Fig. 5 shows the sedimentation pattern of RNA extracted from the same purified virus. While the intact virus sedimented rapidly ($s_{20,w}$ = 147 ± 5) with a sharp boundary, the RNA sedimentation revealed a heterodisperse pattern with a leading component of

TABLE X

Effect of Heterologous Nucleic Acid and Nucleic Acid Components on Infectivity of Type 1 Poliovirus RNA for HeLa Cells

Virus RNA mixed at 0°C. and at pH 7.2 with a 1 per cent (*w/v*) concentration of:*	Per cent of maximum RNA infectivity		
	Experiment 1	Experiment 2	Experiment 3
Buffer (control)	100	100	100
Yeast RNA‡	<1	<1	<1
HeLa cell RNA	<1	<1	<1
Salmon sperm DNA	<1	<1	<1
Alkali-degraded RNA (nucleotides)‡, §	>90	>90	>90
Ribonuclease-degraded RNA (nucleotides and oligoribonucleotides)‡, ‖	<1	<1	<1
Acid-precipitable oligoribonucleotides‡, ¶	<1	<1	<1
Purines, pyrimidines, nucleosides, or nucleotides**	>90	>90	>90
Buffer (but cells treated with 1 per cent yeast RNA before or after exposure to virus RNA)‡‡	>90	>90	>90

* After several minutes of mixing, the mixtures were diluted into 2 M $MgSO_4$ and assayed for remaining infectivity on HeLa cells.

‡ All prepared from 1 lot of 5 per cent yeast RNA.

§ 1 M NaOH, 15 hours at 37° used for hydrolysis.

‖ Digested with ribonuclease for 12 hours at 37°C. Ribonuclease removed by hot phenol extraction; pH adjusted to 7.5 at intervals during digestion of RNA.

¶ Precipitated with cold acid ethanol 5 times.

** Unattached RNA removed by washing prior to addition of virus RNA.

‡‡ Purines, pyrimidines, nucleosides, or nucleotides.

high molecular weight ($s_{20,w}$ = 37 ± 2). Analysis of the sedimentation pattern of the viral RNA with a densitometer indicated that the almost homogeneous rapidly sedimenting component was trailed by slower sedimenting heterodisperse components with an average sedimentation coefficient of $s_{20,w}$ = 24 ± 5. These results indicated that the biological heterogeneity of poliovirus RNA could not be ascribed primarily to extensive breakage of polynucleotied chains during extraction.

Effect of Heterologous Nucleic Acids on Poliovirus RNA.—It was of interest to investigate the inhibitory activity of heterologous nucleic acids reported by

others (20, 21). Table X shows that RNA extracted from HeLa cells, as well as high concentrations of yeast RNA and salmon sperm DNA, strongly inhibited the infectivity of type 1 poliovirus RNA for HeLa cells. Products of complete hydrolysis of RNA caused no inhibition. Alkali-degraded RNA (2′, 3′ nucleotides) and individual purines, pyrimidines, nucleosides, and nucleotides were all ineffective. Depression of poliovirus RNA infectivity by acid-precipitable components from ribonuclease-digested yeast RNA, indicated that inhibitory capacity extended to the level of oligoribonucleotides. Treatment of HeLa cell monolayers with heterologous RNA immediately before or after exposure to poliovirus RNA failed to reduce infectivity. Apparently, heterologous RNA does not inhibit by binding firmly to cell "receptors," at least not so firmly as to resist washing. Since subsequent exposure of cells to heterologous nucleic acid was not inhibitory to RNA infectivity, it is probable that heterologous nucleic acid does not initially act intracellularly, but that it interferes with infectious nucleic acid by formation of an extracellular complex that prevents attachment to, entrance into, and/or replication within cells. In support of this explanation, it has been observed that heterologous nucleic acids do not affect the infectivity of intact poliovirus.

DISCUSSION

Infectivity for HeLa, and other cells, of enterovirus RNA extracted from polioviruses or Coxsackie B1 virus has been assayed reproducibly and with relative sensitivity by employment of (*a*) assay cultures propagated in calf serum medium to strengthen cell attachment to glass, and (*b*) 2 M magnesium sulfate solution as RNA-suspending medium. In our hands, this assay system was superior in sensitivity to monovalent cation methods. Reports of RNA infectivity yields greater than 0.1 per cent of original virus titer accomplished with sodium or potassium chloride media (8, 10, 15), however, suggest that conditions do exist for efficient infection of certain cell lines with RNA in 0.9 M or 1 M monovalent cation solutions in the absence of chelating agents. Moderate susceptibility of mouse brain cells *in vivo* to infectious viral RNA in low salt medium (3) and of cells exposed to foot-and-mouth disease virus RNA in low salt medium (22) further indicate that salt requirements for RNA infectivity may vary with each cellular system.

The influence of salt concentration on enteroviral infectivity for HeLa cells may result from (*a*) alteration of the physical state of RNA before and during entrance into the cell, and (*b*) enhancement of effective incorporation of adsorbed RNA into receptive cellular sites. The effect of salt or hypertonicity on cells might be explained by increased pinocytosis of adsorbed RNA. Enhancement of pinocytosis by salt has been reported for protozoa (23). High salt concentration could be expected to affect viral RNA by induction of extensive hydrogen bonding (24). High concentrations of divalent cations also could

act by direct inhibition of cellular ribonuclease and by increasing the resistance of RNA to enzymatic hydrolysis through facilitation of hydrogen bonding (17).

The comparative infectivity of phenol-extracted tobacco mosaic virus RNA is about 0.01 that of the whole virus from which it was derived and when recombined with tobacco mosaic virus protein it is about 0.5 that of whole virus (25). Since successful recombination of enterovirus RNA with its protein has not been accomplished, only such factors as may be responsible for infectivity of the free RNA will be considered. The rapid adsorption of infective RNA despite failure of cells to adsorb a significant portion of the total enterovirus RNA, as indicated by radioactive label, suggests alternatively that extracted RNA contains a distinctive infectious fraction or that only a small fraction of the RNA successfully initiates infection while the larger unsuccessful fraction of RNA undergoes concurrently inactivation and release into the medium.

The large ultraviolet absorption increment found for RNAase-treated enterovirus RNA and the large proportion of material sedimenting uniformly at 37 S suggest that a significant amount of the phenol-extracted RNA is not badly degraded. Low infectivity of the free RNA cannot be directly attributed to extreme degradation. It is known, however, that the physical particle-plaque forming ratio for whole poliovirus is high (26). If the large fraction of inactive "native" nucleic acid is responsible, physical methods could not be expected to detect the small fraction of infectious nucleic acid. Finally, if the total extracted RNA contains only a small infectious fraction, then this infectious RNA fraction may be associated with a material which facilitates cellular adsorption and infection, and which is lacking in the bulk of the RNA. That such material is not unaltered surface protein of the virus, still retaining specific affinity for cell receptors, is attested by the equally efficient adsorption of infectious RNA by naturally insuscepible L strain mouse cells which lack receptors for adsorption of intact poliovirus.

The physical data obtained with intact poliovirus particles are in agreement with the data of Schwerdt and Schaffer (26) and the physical data with viral RNA are consistent with what would be expected if the RNA were released by phenol extraction as a single intact unit. The greater hypochromicity of RNA within intact poliovirus, as compared to the same RNA extracted from the virus indicates that the RNA is hydrogen bonded within the virus protein coat, as has been shown for bushy stunt virus and other nucleoproteins by Bonhoeffer and Schachman (27). The greater absorbance of the extracted RNA shown in Fig. 3 can be attributed to the use of 0.02 M phosphate as suspending medium. At this ionic strength at pH 7.2 maximal hydrogen bonding of RNA would not occur.

The following paper supports the possibility that some extraneous material must be associated with viral RNA in order for it to be infectious.

SUMMARY

A relatively sensitive and adequately reproducible assay of infectious enteroviral RNA was obtained by exposing calf serum–grown HeLa cells to RNA suspended in 2 M magnesium sulfate solution. Highly purified enteroviral preparations yielded RNA exhibiting more than 0.1 per cent of the infectivity of whole original virus and infectivity regressed linearly with dilution.

Radioisotope experiments with P^{32}-labeled RNA and spectrophotometric studies demonstrated that Gierer-Schramm phenol extraction permits almost quantitative recovery of high molecular weight RNA from poliovirus. Intact chromatography-purified type 2 poliovirus in the analytical ultracentrifuge showed a sharp boundary and a sedimentation coefficient of $s_{20,w} = 147 \pm 5S$. Phenol-extracted poliovirus RNA exhibited a heterodisperse sedimentation pattern with a large proportion of homogeneous, rapidly sedimenting material having a coefficient of $s_{20,w} = 37 \pm 2S$.

Although the bulk of extracted poliovirus RNA as measured by radiophosphorus labeling was not taken up by cells, the infectious fraction of RNA was adsorbed rapidly by HeLa-cell or L strain mouse fibroblast monolayers, indicating a possible dissimilarity between the bulk of extracted virus RNA and a relatively small fraction responsible for infectivity.

Enhancement of poliovirus RNA infectivity for HeLa cells by high ionic-strength magnesium sulfate solution appeared due partly to an effect of hypertonicity on cells, and partly to an effect of high-concentration divalent cation on RNA itself, but not to enhancement of adsorption. Poliovirus RNA adsorbed by HeLa cells apparently was rapidly received within the cells since it became quickly insusceptible to ribonuclease.

Heterologous nucleic acids and degradation products to the level of oligoribonucleotides inhibited infectivity of poliovirus RNA for HeLa cells. This inhibitory activity appeared due to intermolecular complexing, since exposure of cells to heterologous RNA immediately before or after exposure to virus RNA failed to reduce infectivity.

Ultraviolet absorption spectra demonstrated that the RNA within intact poliovirus is more hypochromic (and thus more extensively hydrogen-bonded) than is the same RNA isolated by phenol extraction, and suspended in 0.02 M phosphate buffer.

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