INFECTIVITY OF RIBONUCLEIC ACID FROM POLIOVIRUS IN HUMAN CELL MONOLAYERS*

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Ribonucleic acid preparations (RNA) of four different animal viruses, isolated by the method of Gierer and Schramm (1 (*a*), (*b*)) have been reported to be infectious; in each instance the documented infectivity of the RNA was demonstrated *in vivo* and the RNA preparations were made from tissues infected *in vivo*. Colter *et al.* have reported infectivity in mice, of RNA from Mengo (2) and West Nile (3) virus-infected cells (*i.e.* Ehrlich ascites tumor) in mice and from type II poliovirus-infected suckling hamster brain (3). Wecker and Schäfer have reported comparable activity of RNA from Eastern equine encephalomyelitis virus-infected mouse brain (4).

Cytopathogenic action occurred occasionally in an unpredictable fashion in tissue culture infected by RNA preparations from tissues of infected animals (3, 4). Rigorous proof that the changes were caused by virus RNA itself and not by intact virus was not available because of the random occurrence of the cytopathogenic action.

The present paper describes results of studies on a phenomenon previously reported (5): reproducible infection of HeLa cell monolayers by RNA prepared from partially purified types I and II polioviruses grown in tissue culture. The type of the intact virus which is produced following invasion of the HeLa cells by RNA corresponds to the type of virus from which the RNA was isolated. The evidence presented supports the conclusion that the RNA itself and not intact virus resistant to phenol, induces the infection of HeLa cells. The results suggest that the tissue culture system used offers a suitable tool for quantitative studies on the factors which influence the efficiency of the RNA infectivity and also for biochemical studies of RNA as a determinant of heritable characters and infectious agent.

Materials and Methods

Virus Source.—In all experiments reported, the source for the preparations of polio virus RNA was virus produced and partially purified by the Cutter Laboratories by step 1 of the Schwerdt-Schaffer method (6). After growing virus in either monkey kidney or human amnion

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cells in monolayer, Cutter Laboratories concentrated the virus 100-fold by methanol precipitation, celite adsorption, and elution. The first eluate is designated E_1; two subsequent ones as E_2 and E_3. E_1 has a higher virus content than E_2 and E_3, but the latter contain less non-viral material. The yield from 10 liters of crude virus was received in our laboratory in 300 ml., 100 ml. of each of the eluates, E_1, E_2, and E_3 in 0.88 M NaCl buffered with 0.04 M sodium PO_4 to pH 7.0 to 7.4. The Mahoney strain of virus was used for type I, and MEF-1 strain for type II poliovirus.

The whole virus titer of E_1 preparations of type I virus grown in human amnion cells has varied from 4×10^9 to 1.1×10^{10}, and in monkey kidney 5×10^9 to 1.5×10^{10} plaque-forming units per ml.; E_1 type II virus grown in amnion cells showed a titer of 2×10^9.

Preparation of Infectious RNA from Intact Poliovirus.—Infectious RNA has been made from type I, E_1 and E_2, and type II, E_1 poliovirus suspensions.

The method described by Gierer and Schramm (1) for isolation of RNA from tobacco mosaic virus was used with a slight modification for preparation of RNA from polioviruses. The phenol (114 gm.) is dissolved in 28 ml. of distilled water; this solution is stored at 4°C. and may be used over a period of several months. To each of two 7 ml. pyrex glass centrifuge tubes in an ice bath, 2.5 ml. of a cold E preparation of poliovirus is delivered. The same volume of water-saturated phenol is added, the tubes are closed with a rubber stopper and the mixtures shaken vigorously by hand for 8 minutes at room temperature. The two phases are separated in 30 seconds at 12,000 R.P.M. (high-speed head of International centrifuge, temperature 0°C.). The water phase is transferred to another pair of centrifuge tubes in an ice bath, each containing 2.2 ml. of water-saturated phenol. The mixture is shaken again vigorously for 4 minutes, centrifuged, and the water phase for the third time shaken with an equal volume of water-saturated phenol for 4 minutes and centrifuged. The total water phase is transferred to a conical 12 ml. centrifuge tube and the phenol is removed by five successive extractions with an equal volume of peroxide-free ether (fresh Mallinckrodt). To remove residual ether, nitrogen is bubbled through the solution at 0°C. for 5 minutes. The pH of the solution is adjusted to pH 6.8 to 7.0 with $NaHCO_3$ with phenol red as the indicator. This preparation will be referred to in the paper as "undiluted" RNA even though there has been slight dilution in adjusting the pH. Modification when used will be described for each experiment.

Mallinckrodt phenol was used for preparation of RNA for most experiments. Some chemical companies add a preservative (hypophosphorous acid) to their phenol; the addition is not always marked on the label. Phenols made by different companies have yielded from the same lot of E_1 poliovirus RNA preparations with different degrees of infectivity. The use of phosphate-buffered water for dissolving the phenol has reduced the differences between phenols from different sources. It may prove advisable to free the phenol from the preservative by successive washings with water or by distillation.

Osmotic shock and dodecylsulfate treatment of poliovirus did not yield detectable amounts of infectious RNA.

Cell Monolayers.—Cell monolayers prepared from HeLa populations derived from a single clone L-5 of Sprunt *et al.* (7) by the method of Puck (8), and from the human amnion cell line (9) were used as tools for studying infectivity of RNA preparations. After growth on a side of a rectangular 6 ounce bottle overlayered with 10 ml. of Puck's complete medium (8) for 6 to 7 days, the cells were suspended by trypsinization (0.025 to 0.05 per cent trypsin), centrifuged for 7 to 8 minutes at approximately 1,000 R.P.M., and resuspended in complete Puck's medium. Approximately 1,000,000 cells were seeded in each Petri dish (60 mm.) and incubated for 1 to 2 days at 35°C. in a closed container gassed with 5 per cent CO_2 for a period sufficient to maintain the pH at approximately 7.2–7.5. A comparison of monolayers of ages varying from 17 hours to 7 days showed no significant difference in susceptibility to infection by RNA. Prior

to seeding with the RNA, the fluid was removed from the monolayer and the cells washed 2 times with 2 ml. of Hanks' solution minus Ca, Mg, and phosphate ions. At each washing an effort was made to remove the fluid completely.

After the plates are seeded with 0.1 ml. of the RNA preparation or whole virus suspension, a period of at least 20 minutes is permitted for adsorption before the agar overlay is added unless specified otherwise. The cultures are then incubated as before for 2 to 4 days before the neutral red-containing agar is added in a second overlay. Two to 5 hours after addition of the neutral red-containing agar cytopathogenic action is recorded.

Agar Overlays.—

A. Agar overlay used after seeding of monolayer: To lactalbumin yeast medium described by Melnick (10), 0.25 per cent glucose and 0.3 per cent tris[1] are added. The pH is adjusted to 7.2–7.4 with 1 N HCl. This basic medium is used as follows:

Component 1. Basic medium + 2 per cent agar.[2]

Component 2. Basic medium + 20 per cent horse serum and 3.5 per cent solution of $NaHCO_3$.[3]

A 4 cc. volume of a 1:1 mixture of components 1 and 2 is added to each monolayer.

B. Neutral red-containing agar overlay: For this purpose equal parts of components 1 and 2 are used but component 2 is modified; the horse serum and $NaHCO_3$ are eliminated and neutral red, 1:5,000 final dilution is added.

Enzymes.—All enzymes were dissolved in Hanks' solution minus Ca, Mg and PO_4 ions.

RNAase, crystallized, bovine origin, Armour Co., Chicago.

DNAase, crystalline desoxyribonuclease, Worthington Biochemical Sales Co., Freehold, New Jersey.

Papain, crystallized, Worthington Biochemical Sales Co.

Chymotrypsin, crystallized, salt-free, bovine origin, Armour Co.

Lysozyme, crystallized, prepared from egg whites, Armour Co.

Preparation of Globulins.—Three pools of serum from *rhesus* monkeys, before (*i.e.* "normal") or after hyperimmunization with polioviruses type I or II, were made. The immune monkeys had received repeated intramuscular injections of type I Brunhilde or type II YSK polio-infected monkey spinal cord and medulla, in adjuvants (11).

Serum from rabbits was obtained before or after repeated intravenous injection of active polioviruses type I or II (12).

The globulins from monkey serum were precipitated at ½ saturation ammonium sulfate; the globulins were obtained from rabbit serum at ⅓ saturation. All operations were carried out at 4°C. The precipitated globulins after standing overnight were recovered after centrifugation and restored to original serum volume in distilled water. The cycle of precipitation and solution was repeated twice and after the third (final) precipitation the globulins were dissolved in Earle's solution. Dialysis of the globulin solutions was carried out against 25 volumes of Earle's solution for 6 hours, and, after a change of dialyzing fluid to fresh Earle's solution, dialysis proceeded overnight.

EXPERIMENTAL RESULTS

Most of the experiments which examined RNA and whole virus for qualitative distinction in their infectivity were performed during the period when

[1] Sigma 7 to 9 buffer grade tris (hydroxymethyl) aminomethane, Sigma Chemical Co., St. Louis.

[2] Noble agar Difco (unwashed).

[3] Abbott $NaHCO_3$ 3.75 gm./50 ml. vial.

reproducible cytopathogenic action could be produced by RNA only in the undiluted form. Some of the factors now known to be responsible for failure to induce infection with diluted RNA will be described later. Even though the undiluted RNA produced so many infectious units that a confluent sheet of cell destruction occurred, the effect of the test substances, ribonuclease, normal monkey serum, and normal and polio-immune globulins, used for demonstrating the qualitative difference between infectivity of isolated poliovirus RNA and intact virus, was virtually an all-or-none phenomenon. Therefore the experiments, though only qualitative for the most part, offer a valid basis for comparison. In any one experiment the same RNA preparation was used in the presence and absence of the test substances.

TABLE I

Infectivity of Poliovirus RNA in HeLa Cell Monolayers*

Experiment No.	No exposure to RNAase		Exposure to RNAase <1 min.	
	No. of plates	Cytopathogenic action	No. of plates	Cypathogenic action
1	10	C‡	8	0
2	14	C	16	0
3	14	C	12	0
4	10	C	10	0
5	8	C	15	0
6	8	60§	8	0

* RNA, Experiments 1 to 5: "undiluted" RNA from E_1 poliovirus type I grown in monkey kidney cells. Experiment 6: "undiluted" RNA from E_1 poliovirus type II grown in monkey kidney cells.

‡ C, cytopathogenic action is confluent and covers most of monolayer.

§ Average number of plaques per plate.

Differentiation between Infectivity of Poliovirus RNA and Infectivity of Intact Virus.—

The effect of various test substances on the infectivity of RNA and of the intact virus from which it was derived, was studied by the following procedure. The RNA, or intact virus, (9 parts) was mixed with the test substance (one part), allowed to stand at room temperature for a period of 1 to 10 minutes, and seeded in 0.1 ml. quantities on each of a series of washed cell monolayers. After a 20 to 30 minute interval at room temperature, the standard agar overlay was added and the cultures were incubated, processed, and observed as described under Materials and Methods.

Comparison of Effect of Ribonuclease on Types I and II Poliovirus RNA and on Intact Virus.—In Table I are listed the results of 6 experiments which examined the effect of ribonuclease on the infectivity of types I and II RNA for HeLa cell monolayers. A comparable series of monolayers was seeded in each experiment with 0.1 ml. undiluted RNA without the enzyme. The confluent

cytopathogenic action seen in all monolayers following seeding with undiluted RNA alone has been completely prevented by exposure to ribonuclease. Samples of agar taken from areas of cytopathogenic action produced by RNA regularly yielded whole virus on passage; the type corresponded to the type from which the RNA was prepared. Agar from areas of comparable size in monolayers seeded with the mixture of RNA and ribonuclease, in which no cytopathogenic action appeared, yielded no virus on passage.

Table II shows that the same concentration of RNAase had no demonstrable effect on the infectivity of intact poliovirus.

In table III are listed the results from experiments designed to determine the smallest concentration of ribonuclease required to prevent completely the

TABLE II

Effect of RNAase on Infectivity of Intact Poliovirus Type I in Cell Monolayers*

Virus	Experiment No.	Negative log$_{10}$ final dilution	No. of plaques/ plate	
			Hanks' solution	RNAase 100 μg./ml. in Hanks' solution
HeLa cell virus	1	5.5	15, 26	28, 33
	2	5.5	70	68
	3	6.0	29	26
	4	6.0	23	23
Amnion cell virus E_1 conc.	5	7.5	29	23
	6	7.5	35	29
	7	7.5	17	15
	8	7.5	25	23
	9	7.5	13, 26	15, 15

* RNAase: Virus in contact with RNAase (or Hanks') for 5 minutes before seeding.

invasion of 0.1 ml. of undiluted RNA, when the duration of exposure is less than 2 minutes prior to seeding on monolayers. It is seen that 0.1 μg./ml. rapidly inactivated practically all of the infectious RNA. To insure prevention of infection by the undiluted RNA in all experiments, it was necessary to use a higher concentration. The high concentration used as a routine at first for testing the action of ribonuclease (100 μg./ml.) was tested in the *H. influenzae* transforming system and was shown not to affect the transforming activity of desoxyribonucleic acid (DNA) controlling streptomycin resistance (13). Therefore, contamination of ribonuclease by desoxyribonuclease was not demonstrable.

Influence of Whole Normal Serum and Normal and Polio-Immune Globulins on the Infectivity of RNA and of Intact Virus.—The demonstration of inactivation of the infectivity of the RNA by normal monkey sera by Colter and his associates (2) in *in vivo* experiments has been confirmed repeatedly in the tissue

culture system. Table IV shows that when the undiluted RNA is exposed to a 1:10 dilution of either a pool of normal monkey sera or to individual monkey

TABLE III

Concentration of RNAase for Rapid Inactivation of Infectivity of RNA in HeLa Cells*

Experiment	RNA	Conc. RNAase	No. of plates	Average No. of plaques/plate
		μg./ml.		
1	"Undiluted"	0	5	Confluent
	"	100	3	0
	"	10	4	0
	"	2	4	0
2	"Undiluted"	0	6	Confluent
	"	100	5	0
	"	10	5	0
	"	2	5	0
3	"Undiluted"	0	8	Confluent
	"	0.1	10	1

* RNA, undiluted RNA—Experiment 1. Grown in monkey kidney cell E_1 poliovirus.
Experiment 2. Grown in monkey kidney cell E_1 poliovirus.
Experiment 3. Grown in amnion cell E_1 poliovirus.

TABLE IV

Effect of Normal Monkey Serum on Infectivity of Type I Poliovirus RNA in Human Cell Monolayers

Experiment No.	RNA alone		RNA + serum 1:10 dilution*	
	No of plates	Total No.‡ of plaques	No. of plates	Total No. of plaques
1	3	3,500§	3	3
2	5	1,000	5	0
3	4	800	4	3
4	12	588	12	10

* RNA exposed to serum for 2 minutes prior to seeding.

‡ Total number plaques in all plates seeded. Count is based on inoculum of 0.1 ml. of undiluted RNA per plate.

§ RNA diluted 1:10 in 1 M NaCl + 0.04 M phosphate and seeded in 0.1 ml. per monolayer.

sera for 2 minutes at room temperature before seeding on cell monolayers, most of the infectivity is inactivated. In Experiment 1, a total of 350 plaques were counted on the three monolayers, each of which was seeded with 0.1 ml. of a 1:10 dilution in hypertonic saline. In Experiments 2 and 3, the total count was

derived from an estimate of 200 plaques on each of the 4 or 5 plates which showed confluent cytopathogenic action after seeding with 0.1 ml. of undiluted RNA. The lower plaque count produced by undiluted RNA in experiment 4 is not understood.

On the premise that normal serum might contain sufficient ribonuclease to inactivate the infectivity of poliovirus RNA, measurements were made of the ribonuclease content of a number of normal monkey sera, normal and immune globulin samples, and a few other test substances by the method of Anfinsen (14). Table V indicates that 11 individual monkey sera and one pool showed ribonuclease in concentrations ranging from 0.6 μg./ml. to greater than 5 μg./ml. A single rabbit serum also showed detectable RNAase activity, but none was found in a 1:10 dilution of one normal horse serum, or in globulin from normal or polio-immune rabbit sera.

In view of the absence of RNAase in globulin, it was possible to demonstrate a further difference between the quality of infectivity due to RNA, on the one hand, and that due to intact virus (15). Table VI shows that intact virus is inactivated by globulin made from homotypic serum, but not by normal serum or RNAase. Infectivity of RNA is inactivated completely by RNAase and almost completely by whole normal serum, but not by globulin made from homotypic serum. A sample of bovine plasma albumin in 5 per cent concentration, as well as lysozyme (100 μg./ml.), a basic protein which is comparable in size of molecule to ribonuclease, has been shown not to influence the infectivity of either RNA or the whole virus.

Effect of DNAase on the Infectivity of the RNA.—Schaffer and Schwerdt (16) have reported that crystalline poliovirus is a nucleoprotein, and that approximately 25 to 30 per cent is RNA. Thus far the presence of DNA in very small amounts has not been excluded. It was of interest, therefore, to study the influence of DNAase on the RNAase-labile infective component of poliovirus. In table VII, Experiments 1 and 2, it is seen that when undiluted RNA was mixed with DNAase in a concentration of 100 μg./ml. for a 1 to 2 minute period, infectivity was not destroyed. The difference found in Experiment 1 is not considered significant. A quantitative measure of the effect of DNAase after a 5 minute exposure in Experiment 3 showed no alteration of the infectivity of RNA.

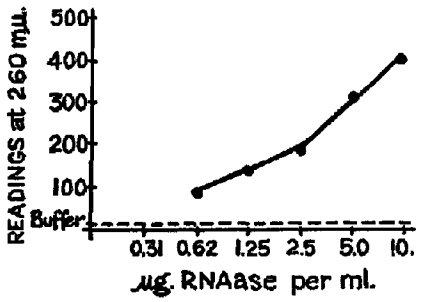
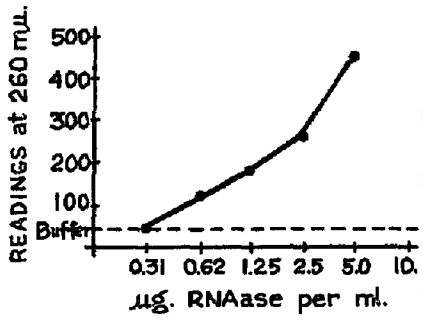
Effect of Chymotrypsin and Papain on the Infectivity of Poliovirus RNA.—Exposure of type I poliovirus RNA to chymotrypsin and papain in a concentration of 100 μg./ml. for a period of 1 to 2 minutes also failed to inactivate infectivity as shown in Table VIII.

Factors which Alter the Degree of Infectivity of Poliovirus RNA for Cell Monolayers.—For several months, during which ribonucleic acid preparations were tested for infectivity, mostly after dilution in Hanks' solution, only occasional plaques formed on HeLa cell monolayers, and their presence was unpredictable.

On passage, the plaque suspension produced intact virus of the type of origin of the infecting RNA, but it was not possible to prove that an occasional intact

TABLE V

Estimation of Ribonuclease Concentrations in Test Substances by Anfinsen Method Using as References the Action of Known Concentrations of Ribonuclease on Yeast Nucleic Acids

Incubation time	Reference curves of action of known concentration of RNAase on yeast nucleic acids	Test substances* "Normal" sera	Readings at 260 mμ.	RNAase estimated
				μg./ml.
25 min.		Monkey		
		1	145	1.4
		2	140	1.25
		3	125	1.1
		4	83	0.6
		5	135	1.2
		Pool A	85	0.6
45 min.		Monkey diluted ½	×2	
		6	40 80	0.45
		7	135 270	2.5
		8	125 250	2.1
		9	840 1680	>5.
		10	140 280	2.7
		11	100 200	1.3
		Rabbit 1	400	4.2
		" globulin	0	<0.3
		Horse 1/10	0	<0.3
		Hanks' solution	0	<0.3

* Test System: *Incubation time at 25°C.*

	25 min.	*45 min.*
Yeast nucleic acid:	0.5 ml. 3.2%	0.33 ml. 0.8%
Test substance:	0.75 ml.	0.5 ml.
Uranyl acetate:	0.25 ml.	0.38 ml.

virus particle could not have survived the phenol action because of the random occurrence of cytopathogenic action. The results of experiments which explored the cause of the low degree of infectivity of the RNA preparation when diluted in physiologic solutions show that the ionic strength of the diluent plays an important role in the invasion of cells by RNA (17).

The poliovirus suspension used for the preparation of the RNA is 0.88 M with respect to NaCl. After three extractions with equal amounts of water-saturated phenol, the water phase is approximately 0.5 M with respect to NaCl.

TABLE VI

Effect of Test Substances on Infectivity of Type II Intact Poliovirus or RNA Isolated from Type II E, Amnion Cell Poliovirus

Test substance (0.1 ml.) mixed with intact virus or RNA (0.9 ml.); 0.1 ml. of mixture per plate

Experiment	Virus or RNA	No. of plates	Average No. plaques/plate						
			RNAase 1000 μg./ml. BSS	Normal monkey serum undiluted	Monkey globulin undiluted			Lysozyme 1000 μg./ml. BSS	Albumin bovine plasma 5 mg./ml. BSS
					Anti-I	Anti-II	Normal		
1	Intact virus $10^{-6.5}$	6 amnion	62	68	55	0	57	65	58
2	RNA	4 amnion	0	10	C*	C	C	C	C
3	RNA	4 HeLa	0	0.8	C	C	C	—	C
4	RNA	6 amnion	—	—	61	61	65	—	—

* C, confluent.

TABLE VII

Influence of DNAase on Infectivity of Type I Poliovirus RNA in HeLa Cells*

Experiment	RNA dilution	Conc. DNAase	No. of plates	Average No. of plaques/plate
		μg./ml.		
1	"Undiluted"	0	8	Confluent
	"	100	8	60
2	"Undiluted"	0	8	Confluent
	"	100	8	Confluent
3	1:50‡	0	6	68
	"	100	6	95

* RNA, preparation source: Experiment 1. E_1 monkey kidney cell poliovirus, type I. Experiments 2 and 3. E_1 amnion cell poliovirus, type I.

‡ 1:50 dilution in 0.9 M NaCl + 0.04 M PO_4.

The RNA preparations produce confluent cytopathogenic action when tested at this stage; when diluted only slightly (1:4) in one of the physiologic salt solutions, the infectivity is greatly decreased. On the other hand, after a 1:10 dilution of the RNA in hypertonic salt solutions, a high degree of infectivity, usually confluent cytopathogenic action, is maintained. Preliminary experiments designed to determine the optimal environment for invasion of the cells

by RNA show that the highest degree of infectivity attainable at present, is obtained when the RNA is diluted in the following solution: 1 M NaCl solution buffered with 0.04 M sodium phosphate at pH 7.2. Table IX lists the results of such an experiment. The interpretation of the other results must await additional studies now in progress. There is a suggestion that some divalent cations

TABLE VIII

Influence of Proteolytic Enzymes on Infectivity of Type I Poliovirus RNA in HeLa Cells*

Enzyme	No. of plates	Cytopathogenic action
Chymotrypsin, 100 μg./ml.	7	Confluent
Papain, 100 μg./ml.	7	Confluent
None	8	Confluent
RNAase, 100 μg./ml.	8	0

* RNA, from E_1 Type I poliovirus grown in monkey kidney cells.

TABLE IX

Influence of Different Salt Environments on Infectivity of RNA in Human Cells in Monolayers*

Diluent	Dilution	No. of plates	Average No. plaques/plate
1. 1 M NaCl + $NaHCO_3$	1:10	3	27 (9–47)‡
2. 1 M NaCl + 0.04 M phosphate	1:10	3	Confluent
3. 1 M NaCl + 0.04 M phosphate	1:10	3	Confluent
+ 8 per cent sucrose	1:100	3	8 (2–17)
4. 7× conc. Hanks' solution + 3% sucrose minus Ca, Mg, and PO_4 ions	1:10	3	7 (0–15)
5. 1:1 of diluents 3 + 4	1:10	3	16 (5–35)
6. Diluent 3 + Mg + Ca ions§	1:10	3	18 (7–26)
7. 1:1 of diluents 4 + 6	1:10	3	6 (5–6)

* RNA from amnion E_1 type I virus.

‡ Numbers in parentheses show variation in plaques/plate.

§ As in Hanks' solution concentrated 10-fold.

inhibit the induction of plaque formation by the RNA and that certain anions like phosphate enhance the reaction.

Recent experiments to be reported in a separate paper (18) show that in appropriate environments the degree of infectivity of the RNA is proportional to the dilution factor. Fig. 1 shows the correlation between dilution factor and plaque counts obtained in one of these experiments.

Time Required for Invasion of Cells by RNA.—Invasion of some cells in the monolayers by poliovirus RNA occurs shortly after contact. In Table X is seen the relationship of duration of exposure of cells to RNA, before the agar overlay is added, to the degree of infectivity of the RNA. When this period is

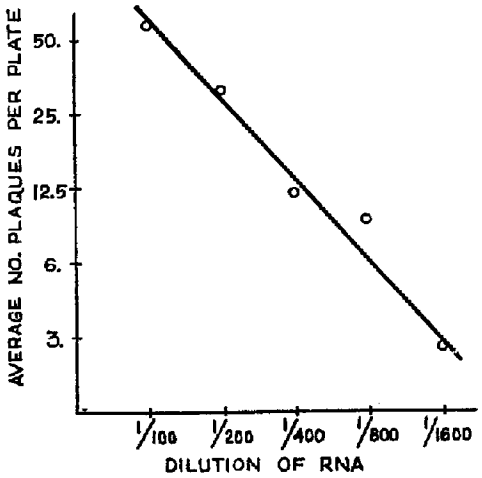


FIG. 1. Correlation of degree of infectivity of poliovirus RNA to dilution factor

TABLE X

Relation of Time of Exposure to "Undiluted" RNA‡ and Degree of Infectivity in HeLa Cell Monolayers*

Experiment	Exposure time	No. of plates	Average No. plaques/plate
1	30 sec.	1	4
	1 min.	3	5
	2 "	1	21
	3 "	2	29
	5 "	1	27
	7 "	1	63
	15 "	6	Confluent
2	2 min.	3	13
	4 "	3	26
	6 "	3	17
	8 "	2	Confluent
	10 "	1	"
	20 "	2	"
	30 "	3	"

* Time of exposure, interval between seeding of RNA on monolayer and addition of agar overlay.

‡ RNA, from E_1 type I poliovirus grown in monkey kidney cells.

varied from 30 seconds to 30 minutes, it is seen that some cytopathogenic action occurs when a 30 second period of contact is allowed, but that the maximum degree recognizable under these conditions occurred later, approximately 10 to 20 minutes after seeding. When the agar overlay is applied earlier than approximately 10 to 20 minutes after seeding of the RNA, some of the poten-

tially infectious RNA is prevented from invading the cells. Whether this is a temperature, ribonuclease, or only a dilution effect is under investigation at present.

DISCUSSION

Ribonucleic acid prepared by the method of Gierer and Schramm from concentrated and partially purified poliovirus types I and II has induced reproducible cytopathogenic action on HeLa and human amnion cell monolayers, yielding intact virus of the type from which the RNA was isolated.

The experimental evidence suggests that it is the poliovirus RNA, itself, and not intact virus particles surviving the phenol action, which is responsible for the cytopathogenic action in cell monolayers. Comparison of the effect of RNAase, whole normal serum, normal and immune globulin on the infectivity of RNA and of intact virus, shows that the infectious agent in the RNA preparation does not exhibit traits of intact virus. Crystalline RNAase and whole serum, (presumably because of the RNAase content), have been shown to destroy the infectivity of RNA, yet show no effect on intact virus. Immune globulin which can completely neutralize whole virus does not reduce the infectivity of RNA preparations. It would seem, therefore, that the virus RNA is the essential infectious agent.

The RNAase used in the experiments reported has been demonstrated to be free of DNAase by showing that it had no inactivating effect on the DNA or transforming principle which induced resistance to streptomycin in *H. influenzae*. Moreover, DNAase in a concentration of 100 μg./ml. did not reduce the infectivity of poliovirus RNA. The possibility that a trace of DNA in purified poliovirus can play the role of a heredity determinant would seem to be excluded.

Two proteolytic enzymes, chymotrypsin and papain, failed also to inactivate the infectivity of poliovirus RNA. This would suggest that viral protein is not needed for induction of the cytopathogenic action in cell monolayers.

Since the concentration of RNA in our preparations was only about 20 to 30 μg./ml., it is not possible at present to prove that the protein content was as low as in comparable material from tobacco mosaic virus. However, our RNA preparations showed a characteristic ultraviolet spectrum.

The degree of infectivity has been shown to be greatly decreased when RNA was diluted in physiologic solutions and to be best maintained by hypertonic salt solutions (1 M NaCl and 0.04 M phosphate to pH 7.2). The data suggest that some divalent cations inhibit the induction of plaque formation by the RNA and that certain anions like phosphate enhance the reaction. When RNA was stored in this solution at -70°C. its infectivity remained for 6 to 8 weeks.

Recent experiments to be reported show that in appropriate environments the infectivity of the RNA is almost 1 per cent of the infectivity of the intact

virus from which it is prepared. The degree of infectivity is proportional to the dilution of the RNA.

Following the invasion of HeLa cells with poliovirus RNA, the cell is directed not only to the replication of the new and specific RNA, but is also induced to produce a new protein needed for elaboration of the intact virus of the specific type from which the RNA had been prepared. These events suggest an analogy with those which follow the invasion of bacteria by DNA, injected by a bacterial virus; the same DNA may play the role of an infectious agent or that of an heredity determinant.

SUMMARY

Ribonucleic acid prepared by the method of Gierer and Schramm from concentrated and partially purified types I and II polioviruses has been demonstrated to be infectious for HeLa and human amnion cells in monolayers. In areas of cytopathogenic action resulting from invasion of cells by RNA, intact poliovirus, of the type from which the RNA had been prepared, is present.

The infectivity of the RNA was completely inactivated by a 2 minute exposure to purified ribonuclease or to whole normal monkey serum shown to contain measurable concentrations of this enzyme. Whole virus infectivity was not influenced by RNAase or whole normal monkey serum. Normal and polio-immune globulin, desoxyribonuclease, lysozyme, proteolytic enzymes, and bovine albumin failed to inactivate the infectivity of RNA.

The degree of infectivity of isolated RNA from poliovirus for cells in monolayer was greatly influenced by the ionic strength of the environment.

The experimental evidence suggests that isolated poliovirus RNA is the carrier of the biological activity responsible for infection of cells and for transmission of genetic information which controls type specificity.

BIBLIOGRAPHY

1. (*a*) Gierer, A., and Schramm, G., *Z. Naturforsch.*, 1956, **11b,** 138. (*b*) *Nature*, 1956, **177,** 702.
2. Colter, J. S., Bird, H. H., and Brown, R. A., *Nature*, 1957, **179,** 859.
3. Colter, J. S., Bird, H. H., Moyer, A. W., and Brown, R. A., *Virology*, 1957, **4,** 522.
4. Wecker, E., and Schäfer, W., *Z. Naturforsch.*, 1957, **12b,** 415.
5. Alexander, H. E., Koch, G., Mountain, I. M., Sprunt, K., and Van Damme, O., *Virology*, 1958, **5,** 172.
6. Schwerdt, C. E., and Schaffer, F. L., *Virology*, 1956, **2,** 665.
7. Sprunt, K., Leidy, G., and Redman, W. M., data to be published.
8. Puck, T. T., Marcus, P. I., and Cieciura, S. J., *J. Exp. Med.*, 1956, **103,** 273. Puck, T. T., Cieciura, S. J., and Fisher, H. W., *J. Exp. Med.*, 1957. **106,** 145.
9. Fogh, J., and Lund, R. O., *Proc. Soc. Exp. Biol. and Med.*, 1957, **94,** 532.
10. Melnick, J. L., *Ann. New York Acad. Sc.*, 1955, **61,** 754.

11. Sprunt, K., Mountain, I. M., Redman, W. M., and Alexander, H. E., *Virology*, 1955, **1**, 236.
12. Mountain, I. M., *Proc. Soc. Exp. Biol. and Med.*, 1955, **90**, 271.
13. Alexander, H. E., and Leidy, G., *J. Exp. Med.*, 1953, **97**, 17.
14. Anfinsen, C. B., Redfield, R. R., Choate, W. L., Page, J., and Carroll, W. R., *J. Biol. Chem.*, 1954, **207**, 201.
15. Mountain, I. M., Alexander, H. E., Koch, G., Sprunt, K., and Van Damme, O., *Bact. Proc.*, 1958, 84 (abstract).
16. Schaffer, F. L., and Schwerdt, C. E., *Proc. Nat. Acad. Sc.*, 1955, **41**, 1020.
17. Koch, G., Alexander, H. E., Mountain, I. M., Sprunt, K., and Van Damme, O., *Fed. Proc.*, 1958, **17**, 256 (abstract).
18. Koch, G., Koenig, S., and Alexander, H. E., data to be published