ON THE ROLE OF RIBONUCLEIC ACID IN ANIMAL VIRUS SYNTHESIS

II. Studies with Ribonuclease*, ‡

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Evidence has been presented in the preceding communication (1) indicating that cellular ribonucleic acid (RNA) plays an important role in the reproduction of deoxyribonucleic acid (DNA)–containing animal viruses. It was shown that 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)[1] inhibits uptake of labeled adenosine into RNA of cells when present in concentrations inhibitory for vaccinia or adenovirus multiplication.

The results of the present study with ribonuclease (RNase) and vaccinia and herpes simplex viruses have corroborated and expanded the results obtained with DRB.

Le Clerc (2) was the first to show that RNase inhibits the multiplication of an animal virus. Influenza A virus multiplication in the chorioallantoic membrane of embryonated chicken eggs was inhibited by crystalline bovine pancreatic RNase both *in vitro* and *in vivo*. RNase had no direct inactivating effect on influenza virus particles, and membrane tissue, the capacity of which to produce influenza virus had been markedly reduced by RNase, recovered this capacity after washing and incubation at 37°C. RNase inhibited influenza virus multiplication only when given within 2 hours after virus inoculation. It was also shown that RNase was capable of inhibiting adenine-C[14] incorporation into membranes. The original observation (2) that RNase inhibits influenza virus multiplication in the chorioallantoic membrane has been confirmed (3).

The combined inhibitory effect of RNase and DRB on influenza B virus multiplication will be described. Inhibition by RNase of vaccinia virus multiplication in the chorioallantoic membrane *in vitro* and of pock formation *in vivo* will be reported. The relationship between time of administration of RNase

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and inhibition of vaccinia and influenza virus multiplication will be described. Inhibition of herpes virus pock formation *in vivo* by RNase will also be reported. It will be shown that RNase has no direct inactivating effect on the infectivity of vaccinia and herpes viruses and that its effect on pock size is slight.

Materials and Methods

Culture Medium.—The buffered glucosol medium employed for cultures of chorioallantoic membrane was described in the preceding communication (1). This medium was also used as the diluent for RNase in both *in vitro* and *in vivo* experiments.

Buffered Saline.—The composition was given in the preceding communication (1).

Viruses.—The vaccinia virus strain used was obtained as a calf lymph preparation from the New York City Department of Health. The material employed in the present study represents the fifth chorioallantoic membrane passage of the calf lymph stock. Two seed virus stocks were prepared and stored in a manner described previously (4). The stocks contained, respectively, 3.4×10^9 and 1.9×10^9 pock-forming units (PFU) per ml. of chorioallantoic membrane.

The herpes simplex virus used was an egg-adapted variant of the HF strain of herpes virus obtained from Dr. Thomas F. McNair Scott. The chorioallantoic membrane of 12-day-old embryonated eggs was inoculated ectodermally with 31 PFU of herpes virus per egg. The virus inoculum was contained in 0.2 ml. After 48 hours' incubation at 35°C. the infected portions of the chorioallantoic membrane were collected and a 10 per cent suspension by weight was prepared in buffered saline containing 5 mg. of gelatin per ml. This suspension was homogenized in a modified Waring blendor at 4°C. and centrifuged at 400 g for 5 minutes. The supernatant was collected, distributed in nitrocellulose tubes, frozen, and stored at -55°C. until used. The seed virus used in the present studies contained 2.9×10^8 PFU per ml. of chorioallantoic membrane.

Preparation of Embryonated Eggs for Experiments or Infectivity Titrations on the Ectodermal Surface of the Chorioallantoic Membrane.—The ectodermal surface of the chorioallantoic membrane of embryonated eggs was prepared for inoculation according to the technique of Nadeje *et al.* (5).

The embryonated eggs whose chorioallantoic membrane had been "dropped" were used for experiments on pock formation by vaccinia and herpes viruses and for determination of concentration of these viruses by the pock-counting technique. For these purposes virus-containing materials were placed on the ectodermal surface of the "dropped" chorioallantoic membrane. The volume inoculated was 0.2 ml. unless otherwise specified. The needle used to introduce the inoculum was moved from side to side to distribute the inoculum over the exposed ectodermal surface of the chorioallantoic membrane. In most experiments the inoculated eggs were incubated at 35°C. for 48 hours; in some experiments a longer incubation period was employed. At the end of the incubation period, the eggs were cut along their ellipsoidal axis, the infected portion of the choriorallantoic membrane was collected and pocks formed on the ectoderm were examined and counted.

Cultures of Chorioallantoic Membrane.—The procedure of preparation was described earlier (6). These suspension cultures of surviving tissue were used for experiments on multiplication of influenza and vaccinia viruses. With influenza virus the yield was measured in the medium whereas with vaccinia virus it was determined in the membrane extract. This was done be-

cause influenza virus is almost completely liberated from the chorioallantoic membrane into the medium (7) whereas vaccinia virus is not released from the membrane to a significant extent (4).

Hemagglutination Titrations.—Concentration of influenza virus was determined by hemagglutination titrations. The technique used was described previously (6). The yield was expressed in hemagglutinating units per milliliter. In chorioallantoic membrane cultures 3.8 cm.^{2} of membrane was present per ml.

Infectivity Titrations.—Concentration of vaccinia virus was determined by enumeration of pocks on the ectodermal surface of the dropped chorioallantoic membrane. The method used was described previously (4). The yield was expressed in pock-forming units per milliliter. In chorioallantoic membrane cultures the amount of membrane per ml. was 3.8 cm.^{2}.

EXPERIMENTAL

Inhibition of Lee Virus Multiplication by RNase.—The inhibitory activity of three preparations of pancreatic RNase on Lee virus multiplication was determined in the chorioallantoic membrane *in vitro*. Two preparations were commercial samples. A sample of Armour and Co. RNase was supplied by Dr. Le Clerc. The second was secured from General Biochemicals, Inc. (GBI). The third was a chromatographically pure sample of ribonuclease prepared and supplied by Dr. C. H. W. Hirs.

Cultures of chorioallantoic membrane from 10 to 11 day embryonated chick eggs were set up in 40 x 200 mm. tubes. Each tube contained 23 cm.^{2} of chorioallantoic membrane suspended in 6.0 ml. of buffered glucosol with or without RNase. Concentration of RNase was varied over a wide range. The inoculum consisted of 1.3 $\times$ 10^6 EID$_{50}$ of Lee virus per ml. The tubes were stoppered tightly and incubated at 35°C. for 41 hours with horizontal shaking. Yield of virus was determined in the medium by the hemagglutination technique.

The results of experiments recorded in Table I show that the three preparations of RNase possessed similar activity in inhibiting Lee virus multiplication. At a concentration of 12.5 μg. per ml. RNase caused 52 per cent reduction in yield of virus. At 50 and 200 μg. per ml. the reduction was 66 and 92 per cent respectively. The relationship between concentration of RNase and virus yield is illustrated in Fig. 1. Although inhibition of virus multiplication was obtained at low concentrations of RNase, inhibition was not complete at high concentrations. These findings are somewhat at variance with those reported earlier (2). Le Clerc used several hundred micrograms of RNase in both *in vitro* and *in vivo* experiments, and obtained more than 99 per cent inhibition of PR8 virus multiplication in both. According to Dr. Le Clerc (8) the preparation of GBI RNase which was used in her experiments was more active than the Armour preparation employed in the present study.

Inhibition of PR8 Virus Multiplication by RNase.—The inhibitory activity of RNase on Lee and PR8 virus multiplication was compared.

TABLE I
Inhibition of Lee Virus Multiplication by Ribonuclease (RNase) in the Chorioallantoic Membrane (CAM) in Vitro

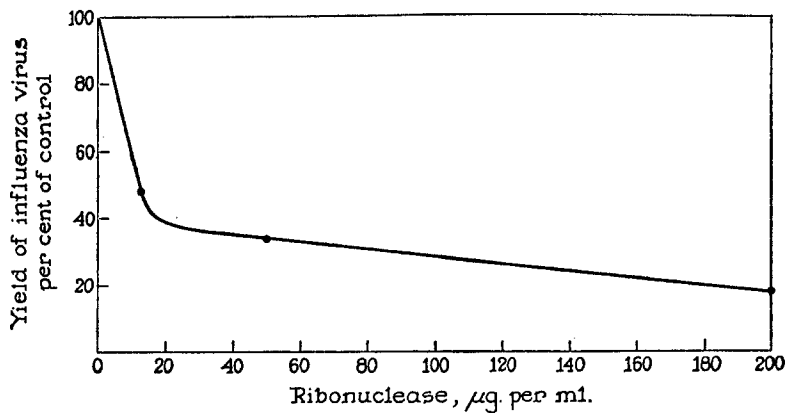| Experiment	Preparation of RNase	Yield of virus, *hemagglutinating units per 3.8 cm.^{2} of CAM*			
		RNase, $\mu g./ml.$			
		None	12.5	50	200
A	Armour	32	10	6.3	3.0
B	Armour	76			12
	"	78		16	
C	Armour	79	18		
D	GBI	158	71	50	25
E	Armour	126		63	
F	Hirs	100	71		
G	Hirs	100			25
	"	133		30	
	GBI	100			20
	"	133		38	
	Armour	100			21
	"	133		34	
H	Hirs	119	79		
	Armour	89	40		
Mean............		99	48	34	18
Inhibition, *per cent*.		0	52	66	82

FIG. 1. Relationship between concentration of RNase and yield of influenza virus in the chorioallantoic membrane *in vitro*. Each point represents the mean of 6 or more determinations.

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Cultures of chorioallantoic membrane were prepared and used in a manner described above and the inoculum was 1.3×10^6 EID$_{50}$ of Lee or PR8 virus per ml.

RNase (Armour) at a concentration of 50 μg. per ml. caused 79 per cent inhibition of multiplication of Lee virus and 84 per cent inhibition of that of PR8 virus. Thus, the activity of RNase was similar with both viruses.

TABLE II

Combined Effect of Ribonuclease (RNase) and 5,6-Dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) on Lee Virus Multiplication in the Chorioallantoic Membrane in Vitro

Experiment	Yield of virus							
	Control		DRB		RNase		DRB + RNase	
			40 μM		12.5 μg./ml.		40 μM + 12.5 μg./ml.	
	HAU*	Inhibition	HAU*	Inhibition	HAU*	Inhibition	HAU*	Inhibition
		per cent		*per cent*		*per cent*		*per cent*
A	79	0	7.9	90	18	77	2.5	97
B	126	0	32	75	63	50	12	90
Mean....		0		83		64		94
			27 μM		8.3 μg./ml.		27 μM + 8.3 μg./ml.	
C	188	0	119	37	63	66	32	83

* Hemagglutinating units.

Combined Effect of RNase and DRB on Lee Virus Multiplication.—On the basis that both RNase and DRB are capable of disturbing RNA metabolism it would be expected that their combined inhibitory effect would exceed the inhibitory effect of either alone. This possibility was examined experimentally.

Cultures of chorioallantoic membrane were prepared and used in a manner described above. The inoculum of Lee virus was 1.3×10^6 EID$_{50}$ per ml.

Results of three experiments are summarized in Table II. In experiments A and B the final concentration of DRB was 40 μM and that of RNase (Armour) was 12.5 μg. per ml. The mean of these two experiments indicates that DRB alone caused 83 per cent, RNase alone 64 per cent, and DRB plus RNase 94 per cent inhibition. Experiment C, in which lower concentrations of DRB and RNase were employed, gave a similar result.

Inhibition of Vaccinia Virus Multiplication by RNase in the Chorioallantoic Membrane in Vitro.—The effect of RNase on the multiplication of vaccinia virus in the chorioallantoic membrane *in vitro* was determined.

Chorioallantoic membrane was obtained from 10-day embryonated eggs. Each culture tube contained 23 cm.^{2} of chorioallantoic membrane suspended in 6.0 ml. of buffered glucosol medium. The inoculum consisted of 7.7 × 10^6 PFU of vaccinia virus per ml. Virus and RNase were introduced simultaneously. The concentration of RNase was 12.5, 50, or 200 μg. per ml.; control cultures received virus but no RNase. The cultures were incubated for 3 hours at 35°C. with shaking at 80 oscillations per minute. The membranes were then washed twice
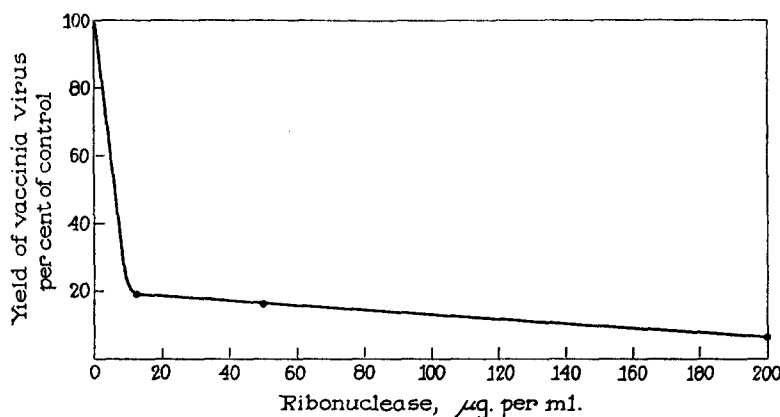

Fig. 2. Relationship between concentration of RNase and yield of vaccinia virus in the chorioallantoic membrane *in vitro*. Each point represents the mean of two determinations.

in 15 ml. of medium, suspended in 5 ml. of fresh medium or RNase solution, and reincubated for a total of 48 hours. The membranes were then homogenized in glass grinders with 12 ml. of fresh medium. The homogenates were centrifuged at 400 g for 5 minutes and the supernatants were collected and stored at −55°C. The yield of virus was determined by enumeration of pocks on the dropped CAM of embryonated chicken eggs and expressed in PFU per 3.8 cm.^{2} of CAM.

Results obtained in two such experiments are summarized in Table III. As can be seen, RNase (GBI) at a concentration of 12.5 μg. per ml. caused 81 per cent reduction in yield of vaccinia virus. At 50 and 200 μg. per ml. the reduction was 84 and 94 per cent respectively. The relationship between concentration of RNase and virus yield is graphically illustrated in Fig. 2. It is evident that inhibition of virus multiplication was obtained at low concentrations of RNase. However, complete inhibition was not obtained at the highest concentration used, which was 27 times greater than the 50 per cent inhibitory concentration. These findings are closely similar to the results obtained with influenza virus.

Inhibition of Vaccinia and Influenza Virus Multiplication in the Chorioallantoic Membrane Relative to Time of Addition of RNase.—To determine whether RNase would inhibit vaccinia virus multiplication after infection had occurred, the enzyme was given at different times after inoculation of virus. A similar experiment was carried out with influenza virus.

In both experiments the membrane cultures were incubated at 35°C. for 48 hours. In the experiment with vaccinia virus the inoculum consisted of 7.7 × 10⁶ PFU of virus per ml. and the membranes were washed at 3 hours. RNase was added to a final concentration of 12.5

TABLE III

Inhibition of Vaccinia Virus Multiplication by Ribonuclease (RNase) in the Chorioallantoic Membrane (CAM) in Vitro

Experiment	Part	Yield of virus, $PFU/3.8$ $cm.^2$ of $CAM \times 10^{6}$ *			
		None	RNase, $\mu g./ml.$		
			12.5	50	200
A	1	7.3			0.42
	2	3.6			0.24
Mean...........................		5.5			0.33
B	1	15	3.0	2.5	
	2	14	2.5	2.3	
Mean........................		15	2.8	2.4	
Inhibition, *per cent*...............		0	81	84	94

* Means of two titrations.

$\mu g.$ per ml. at the time of virus inoculation or 10 or 24 hours later. Membranes were collected 48 hours after virus inoculation, homogenized, and the virus was measured by pock counts on the dropped chorioallantoic membrane of embryonated chicken eggs.

In the experiment with influenza virus 1.3 × 10⁶ EID_{50} of virus was inoculated per ml. and RNase was added to a final concentration of 50 $\mu g.$ per ml. at the time of virus inoculation or 3, 10, or 24 hours later. Culture medium was collected 48 hours after virus inoculation and the amount of virus present was determined by hemagglutination titrations.

In both experiments, control groups were included which received a volume of culture medium equal to that of RNase solution which was added to experimental groups. The yield of virus in the several control groups employed with each virus showed no systematic variation as a function of addition of 0.6 ml. of control medium at various intervals after virus inoculation and therefore the mean yield in controls was used in computing per cent inhibition in treated cultures.

The results of these experiments are summarized in Table IV. As can be

TABLE IV

Inhibition of Vaccinia and Influenza Virus Multiplication in the Chorioallantoic Membrane (CAM) in Vitro Relative to Time of Addition of Ribonuclease (RNase)

Virus in culated	Addition of RNase			Virus yield at 48 hrs.	
		Concentration	Time	Amount/3.8 cm.² of CAM	Inhibition
		μg./ml.	*hrs.*	*PFU* × 10⁵*	*per cent*
Vaccinia	None	—	—	39	0
	RNase	12.5	0	8.6	78
	"	12.5	10	8.0	79
	"	12.5	24	14	64
				HAU‡	
Influenza	None	—	—	34	0
	RNase	50	0	4.0	88
	"	50	3	4.2	88
	"	50	10	10	71
	"	50	24	25	26

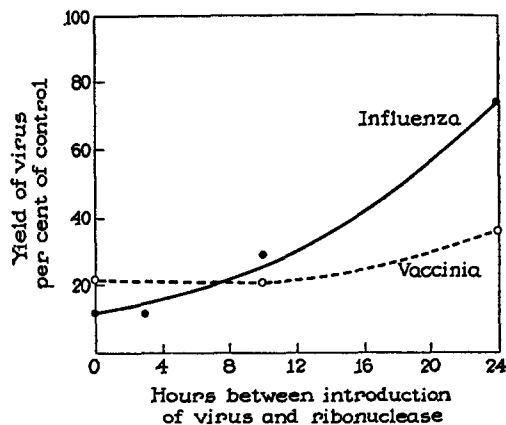* Pock-forming units.
‡ Hemagglutinating units.

FIG. 3. Relationship between time of addition of RNase and yield of vaccinia or influenza virus in the chorioallantoic membrane *in vitro*. Concentration of RNase was 12.5 μg./ml. with vaccinia virus and 50 μg./ml. with influenza virus. Yield was measured at constant time; *i.e.*, 48 hours after virus inoculation.

seen, RNase (GBI) caused marked inhibition of multiplication of both viruses when given 10 hours after inoculation.

The characteristic features of the relationship between time of introduction of RNase and extent of virus inhibition are readily seen in Fig. 3 in which virus

yield is plotted against the interval, in hours, between introduction of virus and RNase. When virus and RNase were given simultaneously the yield of vaccinia virus was reduced by 78 per cent whereas that of influenza was reduced by 88 per cent. This small difference was probably due to the fact that concentration of RNase was 4 times higher with influenza than with vaccinia virus. As was pointed out earlier, the inhibitory effect of RNase on both vaccinia and influenza virus multiplication increased only slightly when concentrations higher than 10 μg. per ml. were used.

With vaccinia virus considerable inhibition was obtained even when administration of enzyme was delayed for 24 hours. In contrast, with influenza virus, little inhibition was obtained when RNase was given 24 hours after virus inoculation.

These results indicate that RNase was inhibitory when given a considerable time after adsorption of the inoculated virus had taken place. However, the results do not provide information as to when RNase acts during a single multiplication cycle because the experiments were of the multiple cycle type. The difference in the inhibitory effect of RNase on vaccinia and influenza virus multiplication, which was observed when the enzyme was given at 24 hours after virus inoculation, was probably due to two factors: (a) The amount of virus taken up by cells under the conditions of these experiments was much greater with influenza (7) than vaccinia (4) virus; consequently the number of cycles of multiplication was probably greater with influenza than with vaccinia virus. (b) The latent period of vaccinia virus multiplication is 2 to 3 times longer than that of influenza virus multiplication. Both of these factors would tend to prolong the interval during which vaccinia multiplication may be expected to be susceptible to inhibition by an agent such as RNase.

Inhibition of Formation of Vaccinia Virus Pocks by RNase on the Chorioallantoic Membrane in Vivo.—Because of the inhibitory effect of RNase on vaccinia virus multiplication in the chorioallantoic membrane *in vitro*, it was of interest to determine whether this enzyme would inhibit formation of vaccinia virus pocks on the ectodermal surface of the dropped chorioallantoic membrane *in vivo*.

Embryonated eggs were prepared as was described above and six eggs were used per variable. The stock suspension of vaccinia virus was diluted 1.4 × 10⁶ times and aliquots of the diluted virus suspension were mixed with equal volumes of RNase solutions of varying concentration. The mixtures were inoculated on the ectodermal surface of the dropped chorioallantoic membrane. Each egg received 0.2 ml. Controls received virus, but no ribonuclease. Eggs were incubated at 35°C. for 48 or 72 hours. The infected portions of chorioallantoic membrane were then collected and the pocks formed were examined and counted.

The results of twelve such experiments with GBI RNase are summarized in Table V. As can be seen, 1.56 μg. of RNase per egg caused 26 per cent reduction in the number of vaccinia virus pocks in eggs incubated for 48 hours and a closely similar degree of inhibition in eggs incubated for 72 hours. With

an amount 130 times greater, *i.e.* 200 µg. of RNase per egg, 92 and 82 per cent inhibition was obtained in eggs incubated for 48 or 72 hours respectively. In two experiments in which the eggs were incubated for 48 hours, 400 µg. of RNase was used per egg and 93 per cent reduction in the pock count obtained.

TABLE V

Inhibition of Formation of Vaccinia Pocks by Ribonuclease (RNase) on the Chorioallantoic Membrane in Vivo

Experiment	Incubation	No. of pocks*								
		RNase, µg./egg								
		None	1.56	3.13	6.25	12.5	50.0	100	200	400
	hrs.									
A		30	22	13						
B		33	13		8.0					
C		26				9.2				
D		15	12							
E	48	8.5	2.5							
F		15	12	13	6.7	5.0	4.7	1.5	1.2	
G		28	25	13						
		34	29	18						
H		28					2.5	4.5	1.3	1.1
		27					4.5	5.4	3.3	2.0
Mean		24	17	14	7.4	7.1	3.9	3.8	1.9	1.6
Inhibition, *per cent*		0	29	42	69	70	84	84	92	93
I		26							5.7	
J	78	26		16	15	9.8				
K		17	17	11	13	12				
L		21		11		5.7	4.8		3.8	
Mean		23	17	13	14	9.2	4.8		4.8	
Inhibition, *per cent*		0	26	43	39	60	79		79	

* Mean of counts on 6 eggs.

The relationship between the amount of RNase and inhibition of pock formation is graphically illustrated in Fig. 4. In the dose range of RNase from 1.56 to 12.5 µg. per egg the inhibitory effect was markedly dependent on the amount of enzyme. Further increase in the amount of RNase had little effect on the degree of inhibition. Even with several hundred micrograms per egg inhibition was incomplete.

The dose-effect relationship observed in these *in vivo* experiments is closely similar to the relationship between RNase concentration and inhibition of

vaccinia or influenza virus multiplication in the chorioallantoic membrane *in vitro*.

In a series of three experiments the activity of several preparations of RNase was compared. The degree of inhibition of pock formation induced by 12.5 µg. of RNase per egg was as follows: two preparations of GBI RNase, 64 and 56 per cent, respectively; Armour, 72 per cent; Sigma, 66 per cent; and Worthington, 69 per cent. Thus the five preparations of RNase possessed similar activity. The mean reduction in pock count was 65 per cent.

Inhibition of Formation of Herpes Virus Pocks by RNase on the Chorioallantoic Membrane in Vivo.—There is cytochemical evidence that the nucleic acid
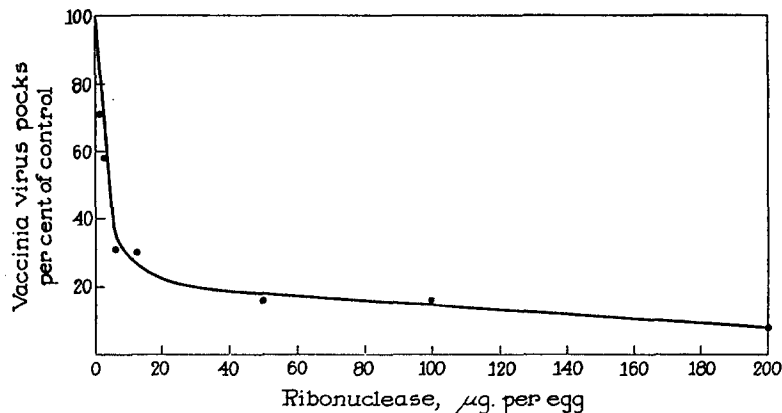

Fig. 4. Relationship between concentration of RNase and number of vaccinia virus pocks on the ectodermal surface of the chorioallantoic membrane *in vivo*. The mean number of pocks per membrane in the absence of RNase, based on 10 determinations, was 24. Each point representing the number of pocks in the presence of RNase is based on 2 to 7 determinations.

in herpes simplex virus is of the DNA type (9, 10). Furthermore, this virus also forms pocks on the dropped chorioallantoic membrane of the embryonated egg. Experiments were therefore undertaken to see whether RNase would inhibit pock formation by herpes virus.

Embryonated eggs were prepared as was described above, and 12 eggs were used per variable. Aliquots of diluted virus suspension were mixed with RNase solutions of varying concentration and the mixtures were inoculated on the ectodermal surface of the dropped chorioallantoic membrane. Each egg received 0.2 ml. Controls received virus, but not RNase. Eggs were incubated at 35°C. for 48 hours either in the usual horizontal position with the false air sac facing upward or in the vertical position with the blunt end of the egg facing upward. The inoculum is distributed over a larger area when the latter procedure is used (11). The advantages of vertical incubation are considerable with herpes virus but they are less significant with vaccinia. After incubation the infected portions of chorioallantoic membrane were collected and the pocks formed were examined and counted.

As can be seen in Table VI RNase (GBI) caused marked inhibition of formation of herpes virus pocks. The effect was more marked when the embryonated eggs were incubated in the horizontal position. Under these conditions the inoculum remained more localized and therefore the dose of RNase per unit area of membrane was higher. It should be emphasized that at comparable virus dilutions confluent lesions were much more common when the eggs were incubated in the horizontal position which made precise quantitation difficult.

TABLE VI

Inhibition of Formation of Herpes Virus Pocks by Ribonuclease (RNase) on the Chorioallantoic Membrane in Vivo

Experiment	Position of eggs during incubation	RNase	Pocks	
			PFU/ml. × 10⁷	Inhibition
		μg./egg		*per cent*
		None	24*	0
		12.5	4.2*	72
		50	1.5	94
		200	1.0	96
A	Horizontal	None	12‡	0
		12.5	2.2‡	82
		50	1.5	87
		200	1.1	91
B	Vertical	None	4.8	0
		12.5	2.3	52
		50	1.7	65
		200	1.1	77

* Confluent lesions considered to be equivalent to 100 pocks.

‡ Confluent lesions not counted.

The results of these experiments are graphically represented in Fig. 5. As can be seen 12.5 μg. of RNase caused a considerable reduction in the number of pocks. However, 200 μg. failed to cause complete inhibition. These characteristics of the dose-effect relationship were also observed with vaccinia and influenza viruses.

In a series of three experiments the inhibitory activity of several preparations of RNase on pock formation by herpes virus was compared. The eggs were incubated in the vertical position. The degree of inhibition induced by 12.5 μg. of RNase per egg was as follows: two preparations of GBI RNase, 46 and 43 per cent, respectively; Armour, 24 per cent; Sigma, 41 per cent; and Worthington, 23 per cent. In the fourth experiment 50 μg. of RNase was employed

per egg and the following results were obtained: two preparations of GBI, 55 and 62 per cent inhibition, respectively; Armour, 61 per cent; Sigma, 71 per cent; and Worthington, 55 per cent. Thus, the inhibitory activity of the five preparations was similar. The mean reduction in pock count with 12.5 μg. of RNase was 36 per cent and with 50 μg. it was 61 per cent.

Lack of Inactivating Effect of RNase on Infectivity of Vaccinia and Herpes Simplex Viruses.—It has been shown that RNase does not cause inactivation of the infectivity of influenza virus particles (2). Experiments were carried out to determine whether RNase had an inactivating effect on vaccinia or herpes virus particles.
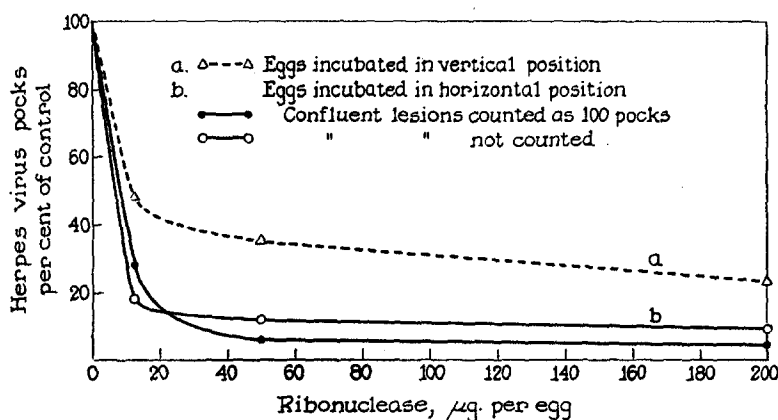

Fig. 5. Relationship between concentration of RNase and number of herpes simplex virus pocks on the ectodermal surface of the chorioallantoic membrane *in vivo*.

Vaccinia and herpes virus seeds, diluted 10^{-2} in buffered glucosol were incubated at 35°C. in the absence or presence of 125 μg. per ml. of RNase. With vaccinia virus the period of incubation was 24 hours. Herpes virus was incubated for 3 hours because of its thermal instability. After incubation the content of infective virus was determined by enumeration of pocks on the dropped chorioallantoic membrane. In the preparation of inocula of suitable concentration for pock counts, concentration of RNase was reduced by dilution below the levels at which it has a detectable effect on pock formation by vaccinia or herpes virus.

Results of experiments summarized in Table VII show that RNase at high concentration did not inactivate the infectivity of vaccinia or herpes virus particles. In these experiments GBI RNase was employed. The effect of Worthington RNase on infectivity of vaccinia virus was also investigated and no inactivation of vaccinia virus infectivity was found.

Effect of RNase on the Size of Vaccinia and Herpes Virus Pocks.—In the course of studies on the effects of RNase on the number of vaccinia and herpes virus pocks, the impression was gained that the pocks which formed in the presence of RNase were on the average somewhat smaller than those in un-

treated controls. This observation was confirmed in experiments in which the membranes bearing pocks were photographed and the pocks measured.

Embryonated eggs were prepared as was described above, and 6 eggs were used per variable. Aliquots of diluted virus suspension with or without RNase were inoculated on the

TABLE VII

Lack of Direct Effect of Ribonuclease (RNase) on Infectivity of Vaccinia and Herpes Simplex Viruses

Virus	Hrs. at 35°C.	Concentration of infective virus	
		Control medium	Medium plus RNase, 125 μg./ml,
		PFU/ml.	*PFU/ml.*
Vaccinia......................	24	1.5 × 10⁹	1.4 × 10⁹
Herpes simplex.................	3	1.0 × 10⁸	1.1 × 10⁸

TABLE VIII

Effect of Ribonuclease (RNase) on the Number and Size of Vaccinia and Herpes Virus Pocks on the Chorioallantoic Membrane in Vivo

Virus	Experiment	Dilution of seed — log	RNase	Position of eggs during incubation	Pocks					
					No. per egg		Inhibition	Diameter		Inhibition
					Control	RNase		Control	RNase	
			μg./egg				*per cent*	*mm.*	*mm.*	*per cent*
Vaccinia	A	7.6	12.5	Horizontal	6.3	4.5	29	1.99	1.62	18.6
	B	7.3	12.5	"	16	7.7	52	2.01	1.95	3.0
	C	7.6	12.5	Vertical	9.2	2.6	72	1.87	1.84	1.6
	D	7.3	50	"	27	14	48	1.87	1.71	8.6
	Mean									8.0
Herpes simplex	A	6.3	12.5	Horizontal	35	14	60	1.04	1.01	2.9
	B	6.6	12.5	Vertical	13	7.8	40	1.02	0.87	14.7
	C	6.3	12.5	"	28	18	36	1.01	0.88	12.9
	D	6.3	50	"	36	12	67	1.10	0.93	15.5
	Mean									11.5

ectodermal surface of the dropped chorioallantoic membrane. Each egg received 0.2 ml. Eggs were incubated at 35°C. for 48 hours in the horizontal or vertical position. The infected portions of the chorioallantoic membrane were then collected and photographed. Photographs of vaccinia virus infected membranes were enlarged 1.73 times whereas those of herpes virus infected membranes were enlarged 3.30 times. The longest and shortest diameters of each

pock were measured, and a mean diameter computed. The mean diameter of all pocks on each egg was then computed, and finally a grand mean for 6 eggs taken.

As can be seen in Table VIII vaccinia or herpes virus pocks formed in the presence of RNase were on the average 10 per cent smaller than those formed in untreated controls. The effect of RNase on the number of pocks was much greater than the effect on pock size. This suggests that RNase had little direct effect on the development of accumulations of leucocytes which form the major pathological feature of pocks.

DISCUSSION

The concept that host RNA may play an important role in virus multiplication was advanced several years ago (12) on the basis of early observations on stimulation of cellular RNA metabolism in certain virus infections and on the basis of findings on virus inhibition obtained with 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB).

That RNA synthesis is stimulated in cells infected with DNA-containing vaccinia virus has been well established. Caspersson and Thorsson (13) found that within a short time after infection of chorioallantoic membrane cells of embryonated chicken eggs with vaccinia virus the nucleoli became enlarged and the content of cytoplasmic nucleotides increased markedly. More recently it was shown (14) that cytoplasmic RNA was considerably increased in HeLa cells infected with vaccinia virus. These findings contribute greatly to knowledge of metabolic alterations which occur in virus-infected cells; however, they do not provide evidence as to whether RNA is necessary for synthesis of vaccinia virus particles.

The demonstration that DRB is inhibitory for vaccinia virus multiplication at the same low concentrations which are inhibitory for multiplication of influenza virus (15), combined with the evidence that DRB inhibits incorporation of precursors into RNA strongly suggested that RNA is, indeed, necessary for vaccinia virus synthesis (1). The results of the present study with RNase provide additional evidence in support of this conclusion. Furthermore, evidence was obtained that herpes virus multiplication is also dependent on RNA. There is cytochemical evidence that herpes virus contains DNA (9, 10). In the preceding communication it was shown that multiplication of the DNA-containing adenovirus in monkey kidney cells was as susceptible to inhibition by DRB as that of influenza virus. Thus, it appears that RNA plays a definite role in the multiplication of three DNA-containing animal viruses: vaccinia, adenovirus, and herpes virus.

As was emphasized above, close similarity was observed in the relationships between concentration of RNase and its inhibitory effects on influenza and vaccinia virus multiplication and on pock formation by vaccinia and herpes viruses. This suggests a common mechanism of action in all cases, and there-

fore it appears likely that even with influenza virus, which contains RNA, RNase may be inhibitory because of its effect on host cell RNA. Although Le Clerc (2) showed that RNase markedly reduced the uptake of adenine-C^{14} into chorioallantoic membranes, her preferred hypothesis of the mechanism of action of RNase was that the enzyme might act on free virus nucleic acid after separation of infecting virus particles into ribonucleic acid and protein inside host cells. That such may be the case is possible. Thus, RNase may inhibit influenza virus multiplication through either or both of the two mechanisms suggested.

It is of great interest that RNase had no effect on incorporation of C^{14}-phenylalanine (2), and that the inhibitory effect of DRB on uptake of C^{14}-L-alanine into proteins of the chorioallantoic membrane was slight (1). These findings suggest that RNase and DRB inhibit the multiplication of DNA-containing viruses not because of a secondary effect on the synthesis of host cell enzymes which are of importance in keeping the host cell and the multiplying virus supplied with energy and precursor materials. It appears, rather, that the mechanism of the inhibitory effect on virus yield may be more direct and may involve some function of cell RNA in the synthesis of virus proteins. The possibility suggests itself that inhibition of virus multiplication may be due to a disturbance in the function of soluble low molecular weight RNA as carrier of activated amino acids. Hoagland *et al.* (16) found that transfer of activated amino acids to soluble RNA was sensitive to RNase. The enzyme acts on the phosphate bridge between terminal adenosine and adjacent cytidine of soluble RNA and as a result terminal adenosine is split off. Such an alteration in soluble RNA would interfere with the transport of activated amino acids which are linked to terminal adenosine of soluble RNA and are carried in this form through further steps in protein synthesis (17). Inhibition of protein synthesis in living onion roots treated with RNase appears to be due to partial breakdown of soluble RNA by the enzyme (18).

If it be true that RNase inhibits virus multiplication through interference with the transport mechanism of activated amino acids to sites of synthesis of virus proteins, it follows that host cell protein synthesis is less sensitive to inhibition by this mechanism because RNase had no effect on uptake of C^{14}-phenylalanine into proteins of the chorioallantoic membrane (2). Indeed, it appears that host protein synthesis in the chorioallantoic membrane is unusually insensitive to the action of both RNase and DRB, because both of these agents do inhibit amino acid uptake into proteins in other systems (1, 19–23).

Certain other possibilities should also be considered to explain the mechanism of inhibitory action of RNase on multiplication of DNA-containing viruses; inhibition may be due to disturbance of the possible template function of high molecular host cell RNA in synthesis of virus proteins. However, too little is known about the template function of RNA in protein synthesis to permit a

detailed consideration of this possibility. Finally, the possibility should be considered that in cells infected with a DNA-virus new RNA is synthesized which plays a specific role in the reproduction of the particular virus, and that the effects of RNase and DRB are due to interference with this new RNA.

SUMMARY

Ribonuclease is a highly active inhibitor of vaccinia virus multiplication *in vitro* in the chorioallantoic membrane removed from embryonated chicken eggs. It is also a highly active inhibitor of pock formation by vaccinia and herpes simplex viruses on the chorioallantoic membrane *in vivo*. Marked inhibitory effects were obtained with 12.5 μg. of RNase. However, complete inhibition was not obtained with several hundred micrograms of the enzyme.

RNase caused no inactivation of the infectivity of vaccinia virus particles but it had a marked inhibitory effect on multiplication of this virus when administered many hours after infection of host cells had occurred. RNase also failed to inactivate the infectivity of herpes simplex virus particles.

The results obtained indicate that ribonucleic acid is necessary for the multiplication of two DNA-containing viruses; *i.e.*, vaccinia and herpes simplex.

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