Effect of Actinomycin D and Guanidine on the Formation of a Ribonucleic Acid Polymerase Induced by Foot-and-Mouth-Disease Virus and on the Replication of Virus and Viral Ribonucleic Acid

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The RNA-dependent RNA polymerase induced in baby-hamster kidney cells by infection with foot-and-mouth-disease virus can be detected as early as 60 min. after infection, which is 60 min. before viral RNA synthesis commences. The time at which the polymerase can first be detected coincides with the latest time at which actinomycin D (50 $\mu\text{g.}/10^7$ cells) or guanidine (1 mg./ 10^7 cells) inhibits virus replication. However, by increasing the concentration of guanidine, viral replication can be inhibited later in the growth cycle, casting doubt on the validity of the hypothesis that guanidine acts specifically on the formation of the viral RNA polymerase.

The isolation of a virus-induced RNA-dependent RNA polymerase from cells infected with small mammalian RNA viruses has been reported by several groups of investigators, using a variety of systems, e.g. mengovirus-L cells (Baltimore & Franklin, 1963), poliovirus-HeLa cells (Baltimore, Eggers, Franklin & Tamm, 1963), encephalomyocarditis virus-ascites cells (Horton, Liu, Martin & Work, 1966) and FMDV-BHK cells* (Polatnick & Arlinghaus, 1967).

We found in preliminary experiments that an induced RNA-dependent RNA polymerase was present in BHK cells infected with FMDV, at least 60 min. before the commencement of virus-induced RNA synthesis. As this delay in viral RNA synthesis after formation of the RNA polymerase was not observed in the other virus systems so far examined, the kinetics of formation of the enzyme were investigated in some detail.

The time at which viral RNA polymerase activity could first be detected corresponded to the latest time at which guanidine (Brown, Martin & Underwood, 1966) or actinomycin D (Black & Brown, 1968) inhibited virus replication. Guanidine is generally regarded as inhibiting viral replication by preventing the formation of the viral RNA polymerase (Caligiuri, Eggers, Ikegami & Tamm, 1965). We show, however, that increasing concentrations of guanidine cause inhibition of virus

replication at later stages of the growth cycle. Reasons for this later inhibitory action and for the inhibitory action of actinomycin D are suggested.

MATERIALS AND METHODS

Virus strains. FMDV (type O and type SAT1) was grown in monolayers of BHK cells (BHK 21, clone 13; Macpherson & Stoker, 1962) and assayed by the plaque method (Mowat & Chapman, 1962).

Chemicals. Labelled ribonucleoside triphosphates and uridine were obtained from The Radiochemical Centre, Amersham, Bucks. Phosphoenolpyruvate (sodium salt), unlabelled ribonucleoside triphosphates and pyruvate kinase were obtained from Sigma (London) Chemical Co., London S.W.6, and guanidine hydrochloride was from British Drug Houses Ltd., Poole, Dorset. Actinomycin D was a gift from Merck, Sharp and Dohme Inc., Rahway, N.J., U.S.A.

Isolation of RNA polymerase. Monolayers of 10^8 BHK cells were infected with FMDV (50 plaque-forming units/cell) and incubated for 30 min. at 37°. The unadsorbed virus was then poured off, Eagle's medium (30 ml.) added and incubation continued to 150 min. after infection. (In the experiment on the kinetics of induced polymerase formation, incubation was terminated at the specific times shown in the Results section.) The cell sheets were washed twice with ice-cold phosphate-buffered saline (Dulbecco & Vogt, 1954) and the cells harvested with a rubber 'policeman'. The cells were homogenized in water (10^8 cells/ml.) in a Teflon-glass homogenizer (ten strokes). Inspection at this stage showed the presence of cell debris and clean intact nuclei only. The cell homogenate was then made 10 mM with respect to tris-HCl buffer, pH 7.6, and 5 mM with respect to MgCl_2 .

* Abbreviations: BHK cells, baby-hamster kidney cells; FMDV, foot-and-mouth-disease virus.

The method of isolation of the enzyme from this stage was varied, depending on whether the total-particle fraction or the large-particle fraction was to be used.

(a) Total-particle fraction. The cell homogenate was rapidly mixed with 5M-NaCl (0.1 vol./vol. of homogenate) and kept at 0° for 10 min. An equal volume of water was added and the mixture was centrifuged at 600g for 10 min. at 0° in an MSE Mistral centrifuge. The supernatant was centrifuged at 80000g for 1.5 hr. in a Spinco model L ultracentrifuge. After removal of the 80000g supernatant, the pellet and centrifuge tube were rinsed with 0.25M-sucrose-1mM-MgCl₂ and the pellet was suspended in sucrose-MgCl₂ (0.2 ml./10⁸ original cells). This suspension was used as the total-particle fraction.

(b) Large-particle fraction. The nuclei and the cell debris were removed by centrifugation of the cell homogenate at 600g for 10 min. at 0° in the MSE Mistral centrifuge. The supernatant was mixed with 5M-NaCl (0.1 vol./vol. of supernatant) and an equal volume of water was added. The mixture was kept at 0° for 10 min. and then centrifuged at 40000g for 15 min. in the Spinco model L ultracentrifuge. After removal of the 40000g supernatant, the pellet and centrifuge tube were rinsed with sucrose-MgCl₂ and the pellet was suspended in sucrose-MgCl₂ (0.2 ml./10⁸ cells originally). This suspension was used as the large-particle fraction.

Assay system. The assay system contained (μ moles): ATP, GTP, CTP and UTP, 0.1 each; tris-HCl buffer, pH 8.1, 25; MgCl₂, 5; phosphoenolpyruvate, 5; pyruvate kinase, 2 μ g.; enzyme preparation, 0.1 ml. (400–600 μ g. of protein); total volume, 0.4 ml. The mixture was incubated for 15 min. at 37°. This reaction mixture (with minor modifications as indicated in the Results section) was used throughout the work. In each incubation one of the ribonucleoside triphosphates was labelled with ¹⁴C (specific radioactivity 5 mc/m-mole). After incubation the reaction mixture was rapidly chilled and 10% (w/v) trichloroacetic acid (5 ml.) was added. The reaction mixture was kept at 0° for 60 min. and the precipitate was washed by centrifugation with 3 $\times$ 5 ml. of 10% trichloroacetic acid and 5 ml. of ethanol-ether (1:1, v/v). The precipitate was dissolved in formic acid, transferred to planchets and dried. The amount of incorporated nucleotide was determined by radioactivity measurements with a Panax automatic counter.

Effect of guanidine on virus and viral RNA replication. Guanidine hydrochloride at different concentrations was added at specific times throughout the growth cycle of the virus and the virus yield at 200 min. after infection was determined by the plaque method. For the determination of viral RNA replication, monolayers of BHK cells (10⁷ cells) in 4 oz. medicine bottles were incubated for 2 hr. in Earle's saline containing actinomycin D (10 μ g./10⁷ cells). The replication of FMDV is unaffected by actinomycin D at this concentration (Brown & Cartwright, 1964; Black & Brown, 1968), although host-cell RNA synthesis is inhibited more than 99%. The cells were infected with virus (100 plaque-forming units/cell) for 10 min. at 37°, and the unadsorbed virus was poured off and replaced by Earle's saline (10 ml.). [³H]Uridine (29 c/m-mole) (10 μ C/bottle) was added to a series of bottles for successive 15 min. periods throughout the growth cycle. The labelling period was terminated by removing the medium and adding 10% trichloroacetic acid (4 ml.) at 0°. After standing for 1 hr. at 0° the tissue was

removed from the glass and washed by centrifugation with 10% trichloroacetic acid (3 $\times$ 4 ml.) and methanol (2 $\times$ 4 ml.). The residual precipitate was dissolved in 0.5 ml. of Hyamine 10X hydroxide (Packard Instrument Co. Inc., La Grange, Ill., U.S.A.) at 37° and counted in a Packard scintillation counter with a toluene-2,5-diphenyloxazole-1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene (1000:5:0.3, v/w/w) system at 20–30% efficiency. In experiments with guanidine, the Earle's saline was replaced by Earle's saline containing guanidine at the specific time, and the labelling procedure was continued.

RESULTS

Polymerase activity in the total-particle and large-particle fractions. Table 1 shows that the microsomal fraction from infected cells incorporated ribonucleoside triphosphate into an acid-insoluble product when incubated with the complete assay system. The omission of one ribonucleoside triphosphate precursor caused a decrease in the quantity of labelled ATP or UTP incorporated into acid-insoluble material. This indicated a dependence of the polymerase activity on the presence of all four ribonucleoside triphosphates. A difference was apparent between the incorporation with labelled ATP and that with labelled UTP. With ATP as the labelled precursor the decrease in the incorporation when only three ribonucleoside triphosphates were present was 74%. When UTP was used as the labelled precursor the corresponding decrease was greater than 90%. This suggested the presence of a contaminating polyadenylate homopolymerase. This was in fact confirmed in the experiments with the large-particle fraction.

Table 2 shows that the large-particle fraction had more polymerase activity/mg. of protein than the total-particle fraction and that a partial separation of the heteropolymerase and the homopolymerase had been achieved. Whereas in the large-particle fraction the homopolymerase/heteropolymerase ratio was 1:10, in the particle preparation isolated from the 40000g supernatant it was 1:3. The total

Table 1. RNA polymerase activity of total-particle fraction

For all experimental details, see the Materials and Methods section.

Experiment	Incorporation of [¹⁴ C]ATP or [¹⁴ C]UTP (pmoles/mg. of protein)	
	Complete assay system	Assay system minus UTP or ATP
A	1710 [¹⁴ C]ATP	450 [¹⁴ C]ATP
B	2000 [¹⁴ C]UTP	20 [¹⁴ C]UTP
Non-infected control	15 [¹⁴ C]ATP	20 [¹⁴ C]ATP
80000g supernatant from infected cells	22 [¹⁴ C]ATP	18 [¹⁴ C]ATP

Table 2. *Heteropolymerase and homopolymerase activities in cell homogenate fractions*

For all experimental details, see the Materials and Methods section.

Cell fraction	Incorporation of [^{14}C]ATP (pmoles/mg. of protein)	
	Complete assay system	Minus UTP, CTP and GTP
Large-particle fraction	7600	760
Particle preparation (after centrifugation at 80000g for 1.5 hr.) from 40000g supernatant	1010	380

Fig. 1. Effect of Mg^{2+} ion concentration on polymerase activity of large-particle fraction. For experimental details, see 'Assay system' in the Materials and Methods section.

Fig. 2. Effect of large-particle concentration on incorporation of [^{14}C]GTP. Experimental details were the same as in Fig. 1.

protein in the large-particle preparation was twice that in the particle preparation from the 40000g supernatant. Thus from Table 2 it can be calculated that 92% of the heteropolymerase was in the large-particle fraction.

Dependence on Mg^{2+} ions, enzyme concentration and the effect of ribonuclease. The effect of varying the Mg^{2+} ion concentration in the assay system on the polymerase activity is shown in Fig. 1. The optimum concentration was 5–7 $\mu\text{moles}/0.4\text{ml}$. (15mM). Fig. 2 shows the effect of varying the amount of enzyme (large-particle fraction) on the incorporation of [^{14}C]GTP into acid-insoluble material. Over the range of enzyme concentrations studied, the incorporation of labelled precursor into acid-insoluble product was directly proportional to the amount of enzyme used. Ribonuclease (50 μg .) decreased the incorporation of [^{14}C]ATP into acid-insoluble product by 42% in a 10min. incubation period and by 50% (compared with the assay system plus bentonite) in a 30min. incubation period (Table 3).

Kinetics of incorporation. A large-particle fraction preparation from cells harvested at 2.5 hr. after infection was used. Fig. 3 shows that for the first 15min. the incorporation of [^{14}C]ATP was linear but that it then decreased. By including bentonite (a ribonuclease inhibitor) in the samples incubated for 30min. and 60min., more [^{14}C]ATP was incorporated into acid-insoluble material, presumably because the bentonite inhibited hydrolysis of the product by ribonuclease.

Kinetics of polymerase formation. Infected BHK cells were harvested at different times after infection and the total-particle fraction or the large-particle fraction was prepared and assayed as described in the Materials and Methods section. Fig. 4 shows that polymerase activity was detectable in the large-particle fraction at 60min. after infection and in the total-particle fraction at 90min.; Fig. 4 also shows that viral RNA synthesis was not detectable until 60min. after the appearance of RNA polymerase activity. Brown *et al.* (1966) showed that, under similar conditions of growth, viral RNA synthesis was not detectable until 120min. after infection.

Effect of actinomycin D on virus replication and viral RNA polymerase formation. Actinomycin D inhibits DNA-dependent RNA polymerase (Reich, Franklin, Shatkin & Tatum, 1962), but is without effect on RNA-dependent RNA polymerases. Table 3 shows that actinomycin D had no effect on the incorporation of [^{14}C]ATP into acid-insoluble material in the assay system used here. The replication of FMDV is inhibited by actinomycin D (50 μg ./10⁷ cells), provided that the drug is added within 60min. of infection; the extent of inhibition is dependent on the time at which the drug is added. For example, there was only 10% of the normal yield of virus if the actinomycin D was added with the virus (Black & Brown, 1968). Fig. 4 shows that polymerase activity was first detectable at 60min. after infection, suggesting that the

Table 3. *Effect of actinomycin D and ribonuclease on the polymerase activity of the large-particle fraction*

For experimental details, see the Materials and Methods section.

Assay system	Time after infection ...	Incorporation of [14 C]ATP (pmoles/mg. of protein)	
		10 min.	30 min.
Complete		3200	3600
Plus bentonite (1 mg./ml.)		—	4800
Plus ribonuclease (50 μ g.)		1900	2400
Plus actinomycin D (5 μ g./ml.)		3400	—

Fig. 3. Time-course of incorporation of [14 C]ATP by the large-particle fraction. Experimental details were the same as in Fig. 1. ●, Bentonite (1 mg./ml.) added; ○, no bentonite added.

Fig. 4. Polymerase activity and viral RNA synthesis during viral replication. Δ , Polymerase activity in total-particle fraction; ●, polymerase activity in large-particle fraction; ■, viral RNA synthesis. Experimental details are given in the Materials and Methods section.

actinomycin D-sensitive step may be important to the formation of the polymerase. The effect of

actinomycin D, added at different times after infection, on the appearance of polymerase is shown in Fig. 5. When added later than 60 min. after infection, actinomycin D had no effect on polymerase formation.

Effect of guanidine on virus multiplication, viral RNA replication and viral RNA polymerase formation. Guanidine has been shown to inhibit the growth of certain strains of FMDV, but not that of others (e.g. type 0 is guanidine-resistant and type SAT 1 is guanidine-sensitive; Pringle, 1964). Table 4 shows that guanidine had no effect on the polymerase isolated from BHK cells infected with the two immunological types of virus used. (At the concentration of guanidine used, the replication of virus type SAT 1 is suppressed.) Table 5 shows that adding guanidine inhibits the formation of RNA polymerase in BHK cells infected with FMDV type SAT 1, but does not affect the formation of RNA polymerase in the cells infected with type 0.

Previous studies (Brown *et al.* 1966) on the effect of guanidine on the replication of FMDV type SAT 1 in BHK cells showed that complete inhibition of virus replication occurred only when the guanidine (1 mg./ 10^7 cells) was added within 60 min. of infection. If the guanidine was added later than 105 min. after infection it had no effect on virus replication. Fig. 6(a) shows the effect of different concentrations of guanidine (0.5–3.0 mg./ 10^7 cells) on viral replication when added at different times during the growth cycle of the virus. It is possible to inhibit the replication of the virus by the addition of guanidine as late as 150 min. after infection by using 3 mg./ 10^7 cells. The relationship between log (guanidine concentration) and the latest time at which it has maximum effect is linear (Fig. 6b). It has been shown (Lwoff, 1965; Brown *et al.* 1966) that guanidine concentrations as high as 4 mg./ 10^7 cells have no effect on cellular metabolism. Figs. 7 and 8 show the effect of guanidine (2 mg. and 4 mg./ 10^7 cells) added at the times indicated, on the synthesis of viral RNA. In infected cells not treated with guanidine, viral RNA synthesis began at 105–120 min. after infection. The rate of synthesis increased up to 165 min. after infection and then decreased. The addition of guanidine had an immediate effect on viral RNA synthesis. When added at 120 min. or 135 min. after infection, guanidine (2 mg./ 10^7 cells) stopped the increase in the rate of viral RNA synthesis immediately (Fig. 7). Guanidine (4 mg./ 10^7 cells) added at 135 min. after infection had no more effect than 2 mg./ 10^7 cells added at the same time. At 150 min. after infection, the addition of guanidine (4 mg./ 10^7 cells) did not completely stop the increase in the rate of viral RNA synthesis. This may have been due to asynchronous infection. Within 15 min. of the addition of guanidine there

Fig. 5. Effect of actinomycin D (50 µg./10⁷ cells) on formation of polymerase in large-particle fraction. Experimental details are given in the Materials and Methods section.

Table 4. *Effect of guanidine on polymerase activity of large-particle fraction*

For experimental details, see the Materials and Methods section.

Assay system	Strain ...	Incorporation of [¹⁴ C]ATP (pmoles/mg. of protein)	
		0 _{VI}	SAT 1
Complete		2040	840
Plus guanidine (0.5 mg./ml.)		2160	800

Table 5. *Effect of guanidine on the formation of RNA polymerase*

For experimental details see the Materials and Methods section.

Virus type	Growth conditions	Incorporation of [¹⁴ C]ATP (pmoles/mg. of protein)	
		Control	In the presence of guanidine
0 _{VI}	Control	4800	
	In the presence of guanidine	4400	
SAT 1	Control	840	
	In the presence of guanidine	85	

was a further marked decrease in the apparent rate of viral RNA synthesis (Fig. 8). There was a similar but less marked decrease in the rate of viral

Fig. 6. Effect of guanidine on FMDV replication. (a). Guanidine (0.5–3.0 mg./10⁷ cells) was added at different times to BHK cells that had been infected with FMDV and the yield of virus was measured after 200 min. incubation: ■, 0.5 mg.; △, 0.75 mg.; ○, 1.0 mg.; ●, 1.5 mg.; ◐, 2.0 mg.; ▲, 2.5 mg.; ▽, 3.0 mg. (b). Time at which guanidine has maximum inhibitory effect. ○, Concentration of guanidine; ●, log (guanidine concentration). For experimental details, see the Materials and Methods section.

Fig. 7. Effect of guanidine (2 mg./10⁷ cells) on the rate of viral RNA synthesis. ●, No addition; ○, guanidine added at 120 min. after infection; △, guanidine added at 135 min. after infection. Experimental details are given in the Materials and Methods section.

RNA synthesis between 165 min. and 180 min. after infection when guanidine (2 mg. or 4 mg./10⁷ cells) was added at 135 min. after infection.

Fig. 8. Effect of guanidine (4mg./ 10^7 cells) on the rate of viral RNA synthesis. ●, No addition; ○, guanidine added at 135 min. after infection; ▲, guanidine added at 150 min. after infection. Experimental details are given in the Materials and Methods section.

DISCUSSION

The properties of the enzyme preparation described in this paper are similar to those reported for RNA polymerases induced by other viruses (e.g. Baltimore *et al.* 1963; Horton *et al.* 1966). There was, however, an optimum Mg^{2+} ion concentration (15mM) for the large-particle preparation whereas, in the work of Polatnick & Arlinghaus (1967) with the same virus-cell system, the dependence on Mg^{2+} ions increased linearly up to 8mM and then slowly up to 18mM. The specific activity of the preparation used here was also considerably higher than that used by Polatnick & Arlinghaus (1967).

RNA polymerase activity was detectable 60 min. before the start of viral RNA synthesis (Fig. 4) and reached 30% of its maximal activity by the time viral RNA synthesis was first detected. In all other reports on virus-induced RNA polymerases, there was no evidence for the presence of the enzyme until immediately before synthesis of viral RNA began. The reason for this difference between our results and the observations of other workers is not clear, although it is possible that in our work the enzyme is first formed in an inactive state and is activated by the isolation procedure or in the assay system.

Black & Brown (1968) suggested that the suppression of virus replication by actinomycin D (50 μ g./ 10^7 cells) was due to the inhibition of a host-cell-dependent step. From the results reported here (Fig. 5) it is possible that the actinomycin D-sensitive step is important in the synthesis of the

virus-induced RNA polymerase. This would be expected if (i) a newly-synthesized host-cell protein is necessary for the activity of the induced RNA polymerase, or (ii) a new transfer RNA is required for the synthesis of the polymerase, or (iii) actinomycin D acts on the translation of viral RNA.

However, the apparent dependence of actinomycin D inhibition on the time of its addition to the infected cells may be an artifact, because we have found that the sensitivity of virus replication to guanidine depends on the concentration of the guanidine as well as on the time of its addition.

It has been accepted that guanidine inhibits the replication of small RNA viruses by specific interference with the formation of the induced viral RNA polymerase (Caligiuri *et al.* 1965). Lwoff (1965) suggested that the inhibition by guanidine is due to its preventing the aggregation of the monomer sub-units of the RNA polymerase into the active enzyme. By using a guanidine-requiring mutant of poliovirus, this guanidine-sensitive step was shown to occupy only 10 min. of the growth cycle. Other workers have favoured the hypothesis that the action of the guanidine is on the translation of the viral RNA.

Earlier studies with the virus-cell system used here (Brown *et al.* 1966) showed that there was complete inhibition of virus replication when the guanidine (1mg./ 10^7 cells) was added within 60 min. of infection, which is the time at which virus-induced RNA polymerase is first detected. At later times of addition, the guanidine had less effect, and there was no inhibition with addition at 105 min. These results are in agreement with the hypothesis that the action of guanidine is due to its specific interference with the formation of the viral RNA polymerase.

By increasing the concentration of guanidine, however, we were able to inhibit virus replication at much later times (Fig. 6a). It is impossible to correlate this result with Lwoff's (1965) hypothesis because (a) guanidine suppresses viral replication over a time range extending from the time of infection to 150 min. after infection and (b) whenever the guanidine is added there must be sufficient to prevent the monomers already synthesized from forming the active enzyme, and also to prevent any monomers subsequently synthesized from forming active enzyme. Thus it would not be possible for guanidine of a certain concentration to be effective only during the early part of the growth cycle (this assumes that the polymerase has a short half-life). Even if the polymerase is stable, guanidine added late in the growth cycle would not affect the polymerase already formed, so that viral replication would not be inhibited.

From Fig. 6(b) we can deduce that, irrespective of the site with which the guanidine reacts, the

number of such sites must increase exponentially. If the transcription of viral RNA at any time depends on the number of viral RNA strands available at that time (i.e. it follows first-order kinetics) we could suggest two possibilities for the action of the guanidine: (1) on the transcription process, or (2) on the translation of viral RNA, if translation can occur on progeny RNA as well as on the parental RNA. Fig. 7 shows that the increase in the rate of synthesis of viral RNA is immediately stopped by adding guanidine, but RNA synthesis still continues. If the site of guanidine action is the start of transcription, then the residual RNA synthesis could be due to the completion of strands already in the process of synthesis. If, however, the site of action is in the translation step, it is possible that RNA synthesis would be stopped by the blockage of RNA by incomplete polypeptide chains. It is thus impossible from the evidence in the present paper to decide which alternative is more likely to be correct. However, Jacobson & Baltimore (1968) showed that viral proteins continue to be synthesized in poliovirus-infected HeLa cells in the presence of guanidine. It is tempting to postulate, therefore, that the site of guanidine action is connected with transcription of the viral RNA. During the preparation of this manuscript Caliguiri & Tamm (1968) presented evidence suggesting that

guanidine interferes with the initiation step of viral RNA replication.

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