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Inhibition of Poliovirus Polymerase by Guanidine In Vitro†

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Extracts from poliovirus-infected HeLa cells synthesized 35*s* viral RNA, replicative intermediate, and double-stranded RNA in vitro. Guanidine inhibited the synthesis of all three species of RNA; production of 35*s* RNA was most sensitive to the inhibitor. Pulse-chase experiments with [^{3}H]UTP indicated that guanidine had no detectable effect on elongation of polynucleotide chains or the release of completed RNA chains from the viral replication complex. Experiments in which short pulses of precursor were used suggest that guanidine blocked the initiation step of RNA synthesis in vitro.

Guanidine selectively blocks growth of many picornaviruses (6, 26), several togaviruses (13), and several plant viruses (10, 29). Although guanidine has been shown to interfere with the incorporation of choline into membranes of infected cells (25), prevent capsid proteins from associating with smooth membranes (30), and inhibit the maturation process (16), the major effect appears to be blockage of synthesis of viral RNA (5, 15, 18). There is some discrepancy among published data concerning the stage of RNA synthesis affected by guanidine (5, 6, 15). Presently, it appears that the initiation of RNA synthesis is inhibited (5, 6) and that the release of completed RNA chains might also be blocked under certain conditions (15).

The unpurified poliovirus polymerase obtained from infected cells is a mixture of viral proteins (4, 27) and endogenous RNA, particularly replicative intermediate (RI) (14, 21). Although guanidine severely blocks the production of viral RNA in vivo, studies have failed to demonstrate an inhibition of polymerase activity in vitro, even at concentrations 50- to 100-fold higher than those generally employed during in vivo studies (2). Experiments with guanidine and cell-free extracts from virus-infected cells are difficult to interpret because a large portion of RNA synthesized in vitro is RI and double-stranded RNA (dsRNA) (2, 23, 31), species that comprise only about 10% of viral RNA observed in vivo. During the past several years relatively pure preparations of picornavirus polymerase that utilize exogenous viral RNA or polyadenylic acid as the template and an oligouridylic acid primer have been obtained (9, 11, 12, 20). However, the effects of guanidine on these purified preparations have not been reported. In the present study, polymerase preparations that contain an endogenous template and produce a high proportion of 35*s* viral RNA were derived from infected cells. Pulse-chase studies in vitro with [^{3}H]UTP as a precursor showed that elongation and release of completed 35*s* viral RNA occurred with equal efficiency in the absence or presence of 2 mM guanidine. Experiments in which short pulses of [^{3}H]UTP were used indicated that guanidine retarded RNA chain initiation.

HeLa S_3 cells (Flow Laboratories) were grown as monolayer cultures in Corning roller vessels to 3×10^8 cells per culture. The medium was Earle minimal essential medium (GIBCO Laboratories) containing 10% newborn calf serum, 50 U of penicillin per ml, and 50 μg of streptomycin per ml. For the preparation of polymerase, cells were infected with 50 PFU of Mahoney poliovirus per cell for 3 h at 36°C and then removed from roller vessels with a 0.25% trypsin solution containing 15 mM EDTA. After low-speed centrifugation and two washes with 200 ml of cold Hanks balanced salt solution, cells were pelleted at low speed, suspended in 2 volumes of lysis buffer containing 20 mM HEPES (pH 7.2; *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid), 1.5 mM $Mg(OAc)_2$, 1 mM dithiothreitol, and 11 mM KCl, and ruptured with a Dounce homogenizer on ice. Nuclei were removed by low-speed centrifugation and washed once with an equal volume of lysis buffer. The original supernatant fluid and the wash fluid were combined, and mitochondria were removed by sedimentation at 8,000 × *g* for 15 min in a Beckman SW56 rotor at 0 to 2°C. Glycerol was added to 15% to the supernatant fluid which was stored in 0.7-ml portions in PRO-VIALS at −86°C. The crude enzyme preparations contained, on the average, 24 ± 3 units of absorbance at 260 nm per ml. For assay of enzyme, equal volumes of polymerase extract

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were combined with equal volumes of a reaction mixture that contained 230 mM Tris-hydrochloride (pH 7.4), 18 mM $Mg(OAc)_2$, 56 mM KCl, 29.7 mM phosphocreatine, 667 μg of creatine phosphokinase per ml, 136 to 170 μM each ATP, CTP, and GTP, 100 μg of actinomycin D per ml, and 2 μM UTP. [^{3}H]UTP (37.5 Ci/mmol; New England Nuclear Corp.) was used at 25 to 70 μCi/ml, and reactions were carried out at 36°C in tightly sealed vials.

For the analysis of in vitro RNA by sucrose gradients, enzyme reactions were terminated by the addition of an equal volume of TEN buffer containing 1.0% sodium dodecyl sulfate, 100 mM NaCl, 1 mM EDTA, and 10 mM Tris-hydrochloride (pH 7.2) to the assay mixtures. Samples (2 ml each) were immediately layered over 36-ml, 30 to 15% (wt/wt) linear sucrose gradients prepared in the same buffer with 0.1% sodium dodecyl sulfate and centrifuged from 17 to 18.5 h at 18,000 rpm in a Beckman SW27 rotor at 20°C. Gradients were removed with a Buchler Auto Densi-Flow peristaltic pump with the probe at the bottom of the tube. Fractions of 0.9 ml were collected with an ISCO 1200 PUP fraction collector, and UV absorption was monitored at a wavelength of 254 mm with an ISCO model UA-5 absorbance unit to identify rRNA. In experiments in which the sensitivity of RNA to RNase was measured, one half of each sample was brought to 0.5 M NaCl and treated for 30 min at 35°C with 20 μg of RNase (bovine pancreatic, electrophoretically pure) per ml to identify dsRNA and the backbone of RI. Macromolecules were precipitated in all samples in the cold with 5 volumes of 2% trichloroacetic acid and collected on GF/A glass fiber filters, and radioactivity was measured in vials with Econofluor scintillation fluid (New England Nuclear Corp.).

To test the effects of guanidine on RNA synthesis, preparations of viral polymerase were incubated at 36°C, and the incorporation of [^{3}H]UMP into acid-insoluble polynucleotide was measured in the absence and presence of 2 mM guanidine (Fig. 1). The incorporation in the absence of inhibitor was linear for approximately 10 min and then decreased. Although the incorporation of [^{3}H]UMP decreased about 50% after 15 min of incubation, linear synthesis continued for another 45 min. Six separate enzyme preparations were examined; in all cases, synthesis of RNA continued for at least 2.5 h, which was the maximum period tested. In the presence of 2 mM guanidine, polymerization was equivalent to the control for 10 to 15 min; thereafter, severe inhibition occurred. In several similar studies, inhibition of RNA synthesis by guanidine averaged approximately 75% in the period between 15 and 90 min of incubation. Although

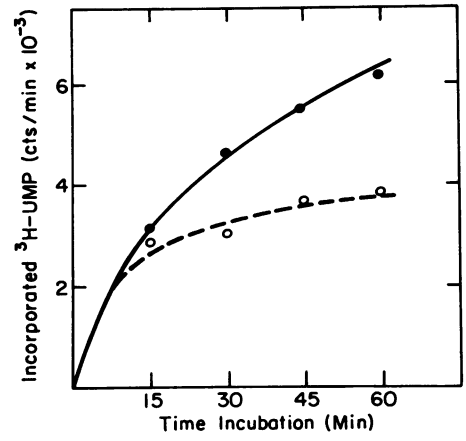

FIG. 1. Effect of guanidine on poliovirus polymerase in vitro. Enzyme was incubated with 70 μCi of [^{3}H]UTP per ml in the absence (●) and in the presence (○) of 2 mM guanidine. Inhibitor was added at the start of incubation, and at intervals 25-μl samples were mixed with 3 ml of cold 5% trichloroacetic acid. Precipitates were collected on GF/A glass fiber filters and washed five times with 15-ml volumes of 2% trichloroacetic acid, and radioactivity was measured in vials containing 5 ml of Econofluor scintillation fluid.

data are not shown, extracts from uninfected cells incorporated only background levels of radioactivity, and enzyme obtained from cells infected with a guanidine-resistant variant of poliovirus was not affected by the inhibitor.

During intracellular growth of poliovirus, about 90% of the viral RNA produced is virion-type RNA with a sedimentation coefficient of 35*s*. The RI and dsRNA species of RNA comprise approximately 10% of the intracellular virus-specific RNA (3, 5). For a determination of whether the crude polymerase synthesized proportionally the same types of RNA that are observed in vivo, products of the in vitro reaction were examined with linear sucrose gradients. RNA produced by the extracts resembled that observed in vivo (Fig. 2). The RI, a large heterogeneous structure, sedimented from approximately 56 to 16*s*; the RNase-resistant 16*s* component represents dsRNA. In this investigation the sedimentation coefficient of the latter ranged from 16 to 18*s*. Variation was probably due to mixtures of dsRNA and RI containing short segments of nascent, single-stranded RNA (3). RNA that sedimented at approximately 4*s* and exhibited resistance to RNase is usually not found in vivo. It could represent fragmented backbone of RI or an abnormal product of in vitro synthesis. The addition of 2 mM guanidine to the assay mixture caused a 65% inhibition of synthesis of 35*s* viral RNA after 90 min of

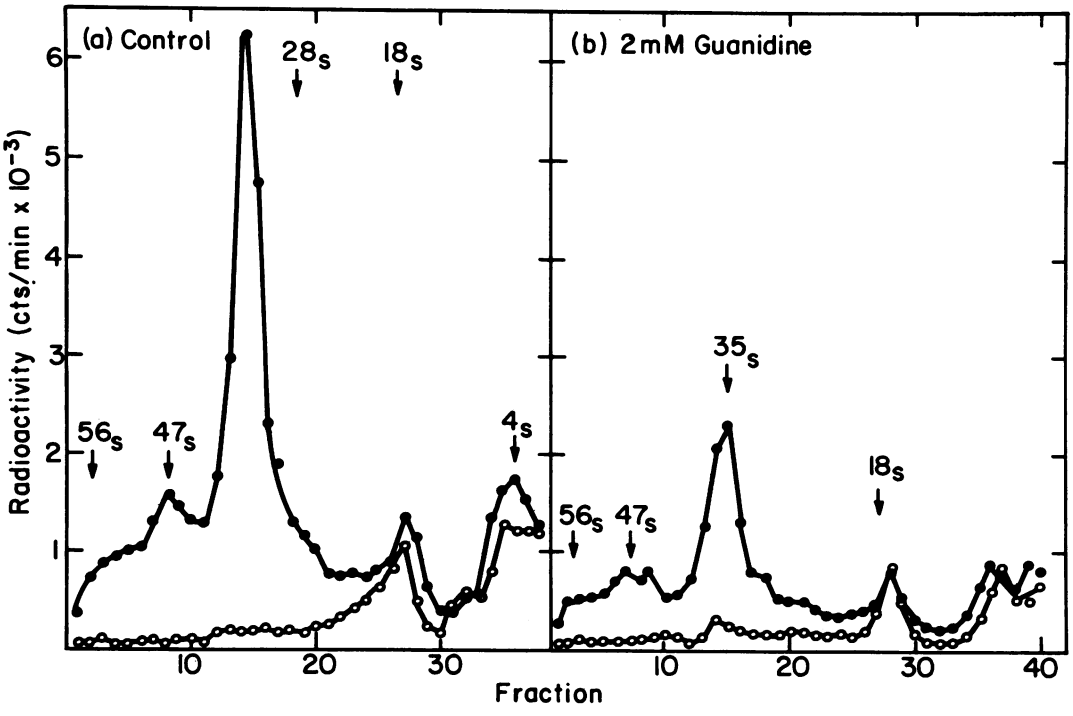

FIG. 2. In vitro product of poliovirus polymerase. Reaction volumes (1 ml each) of polymerase were incubated at 36°C with 25 μCi [^{3}H]UTP for 90 min in the absence (a) or in the presence (b) of 2 mM guanidine. Equal volumes of TEN buffer were added to terminate the reactions, and samples were immediately layered over 36-ml, 30 to 15% sucrose gradients and centrifuged for 18.5 h at 18,000 rpm at 20°C in a Beckman SW27 rotor. After fractionation, each gradient sample was divided in half. The radioactivity was measured in one set without RNase treatment (●) and after incubation with 20 μg of RNase per ml in 0.5 M NaCl (○).

incubation (Fig. 2b). The production of RI and dsRNA was reduced to about 50% of the control levels. This finding is similar to data obtained in vivo that show that the syntheses of RI and dsRNA are more refractory to guanidine than is the synthesis of 35*s* RNA (5).

The step(s) in viral RNA synthesis that is blocked by guanidine in vivo appears to be the initiation of new polynucleotide chains (5, 6) and possibly the release of completed RNA chains from the replication complex (15). The addition of guanidine to cells 3 h after infection (after a pulse with radioactive uridine) does not interfere with elongation or release of completed strands of viral RNA from the replication complex over a short interval; a concomitant reduction in the amounts of radioactive RNA in the viral replication complex occurs (5, 6). This finding is consistent with the view that RNA chains in the process of synthesis are completed and released from the replication complex in the presence of guanidine. At variance with this observation is a report showing an increase in size of the viral replication complex in the presence of guanidine (15). This change is anticipated if the release of mature RNA chains is retarded by the inhibitor. For a determination of which stage of viral RNA synthesis is blocked by guanidine in vitro, reaction mixtures were incubated with [^{3}H]UTP, and after a short pulse, a chase was performed with a 200-fold excess of nonradioactive UTP. In the first series of experiments, [^{3}H]UTP was administered to cell-free extracts during the initial 7 min of incubation; the radioactivity was then chased with unlabeled UTP for 15 min in the absence or presence of 2 mM guanidine. Products of the reaction were analyzed by sucrose gradient centrifugation, and the results of one representative study are shown in Fig. 3a. During the pulse, a heterogeneous population of RNA molecules sedimenting from approximately 60 to 18*s* incorporated the precursor (●). The RNA in the 35*s* region of the gradients is probably completed, single-stranded RNA. After the chase, in the absence of guanidine, 58% of the radioactivity in the small RI (fractions 20 to 30) shifted to both the 35*s* region of the gradient and the larger RI (Fig. 3a, ○). On the average, 24 to 32% of the radioactivity that was lost from fractions 20 to 30 appeared in fractions 5 to 15.

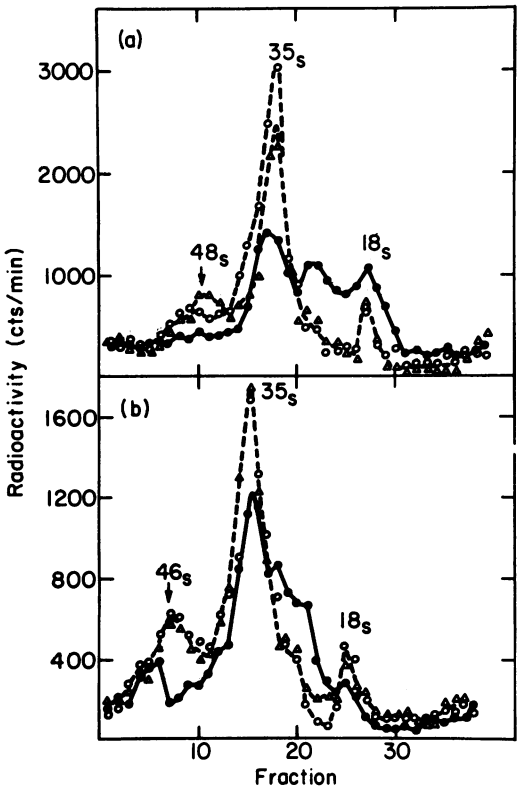

FIG. 3. Effect of guanidine on elongation and release of complete 35*s* viral RNA from the viral replication complex. (a) An enzyme reaction mixture of 2.4 ml was incubated for 7 min at 36°C with 50 μCi of [^{3}H]UTP. One third of the mixture was then combined with an equal volume of TEN buffer containing 1% sodium dodecyl sulfate and placed on ice (●), one third of the sample was incubated for an additional 15 min with a 200-fold excess of unlabeled UTP and then combined with an equal volume of TEN buffer (○), and the final third of the original sample was incubated for an additional 15 min in the presence of a 200-fold excess of unlabeled UTP and 2 mM guanidine (△). Samples were centrifuged for 17 h at 18,000 rpm at 20°C in a Beckman SW27 rotor. (b) The experiment was similar to that of (a) with several modifications. [^{3}H]UTP was added to the enzyme mixture after 10 min at 36°C, and the chase was performed 7 min later in the absence (○) or in the presence (△) of 2 mM guanidine. ●, Pulse. Centrifugation was for 18.5 h at 18,000 rpm at 20°C in a Beckman SW27 rotor.

After the chase, in the presence of guanidine, 60% of the small RI disappeared from fractions 20 to 30 and the radioactivity was redistributed to the 35*s* and large RI regions of the gradient (Fig. 3a, △). The data (Fig. 3) suggest that about 15% more 35*s* RNA was present in untreated controls compared with the guanidine-treated sample after the chase. However, in several identical experiments this was not a reproducible observation. It appears that radioactive RNA was chased from the smaller RI into large RI and 35*s* viral RNA with equal efficiency in the absence or presence of guanidine, a finding in agreement with data obtained in vivo (5).

Because guanidine was inhibitory in vitro during the second stage of synthesis (Fig. 1), pulse-chase studies were also conducted during the latter period. Enzyme was incubated with a complete reaction mixture at 36°C, and 10 min later [^{3}H]UTP was added. After a 7-min pulse, a chase was effected with unlabeled UTP, with or without guanidine. Sucrose gradient centrifugation of the products of the reaction showed that, again, after the chase period 60% of the radioactivity in the small RI was depleted, with the simultaneous appearance of increased radioactivity in the large RI and 35*s* viral RNA (Fig. 3b). The chase was equally effective in the presence or absence of guanidine. In this particular study, guanidine was present only during the chase period, but other studies produced analogous results when guanidine was present throughout the entire experiment. Also, the distribution of RNA throughout the gradient differs from Fig. 3a because a longer centrifugation time was used. Overall, there appeared to be a less effective chase of radioactivity into 35*s* viral RNA in comparison with pulse-chase studies performed during the early stages of the in vitro reaction (Fig. 3a versus b). This might have reflected a proportionally larger amount of 35*s* RNA before the chase shown in Fig. 3b (●). The results indicate that guanidine does not noticeably restrict elongation or release of polynucleotide chains from the viral replication complex during early or later stages of incubation in vitro. This implies that guanidine retards polynucleotide chain initiation. If the initiation of viral RNA synthesis is inhibited by guanidine, a short pulse with [^{3}H]UTP might provide information that would directly support this hypothesis. In an untreated control, [^{3}H]UMP should be incorporated into the large RI, the small RI, and 35*s* RNA as observed in Fig. 3a and b. If initiation were restricted by guanidine, proportionally less of the small RI should incorporate [^{3}H]UMP compared with controls. This assumption was tested by the pulse-labeling of viral RNA in vitro for 4 min after a 25-min preincubation at 36°C. Enzyme in the experimental sample was incubated with guanidine at the start of the incubation. The data from two experiments with separate enzyme preparations are shown in Fig. 4. In the control samples, a short pulse with a precursor resulted in the incorporation of [^{3}H]UMP into RNA that was distributed from 56 to 16*s* of the gradients (fractions 5 to 30). A similar pulse with enzyme incubated with guanidine showed a

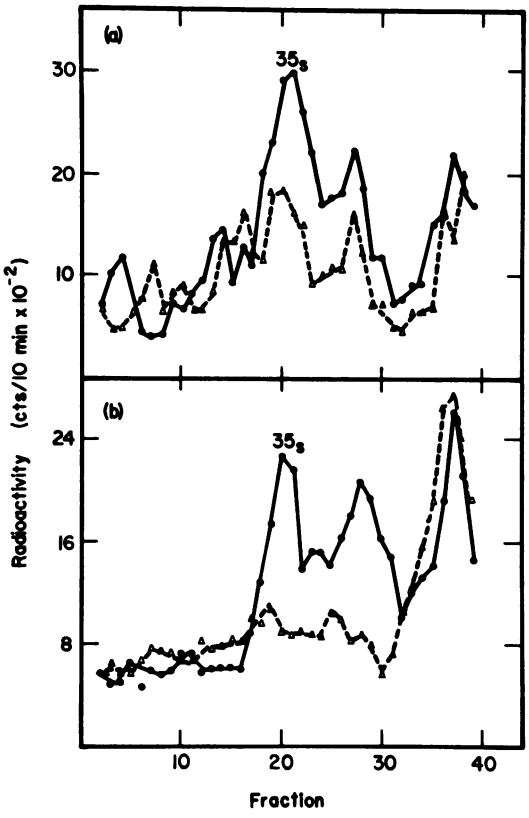

FIG. 4. Effect of guanidine on initiation of synthesis of viral RNA. Two enzyme reaction mixtures of 1 ml were incubated for 25 min at 36°C in the absence (——) or in the presence (------) of 2 mM guanidine. A 25-μCi amount of [^{3}H]UTP was added to each reaction mixture for 4 min. An equal volume of TEN buffer was then added, and the samples were centrifuged for 18 h at 18,000 rpm at 20°C on 30 to 15% sucrose gradients. (a) and (b) represent experiments with two different enzyme preparations.

reduced level of incorporation of [^{3}H]UMP into the small RI (fractions 20 to 30), a result that is anticipated if RNA chain initiation were hindered by the inhibitor. The synthesis of 35*s* viral RNA appears to have been severely blocked in the experiment shown in Fig. 4b. However, the 35*s* region of the gradients contains RI molecules, and the overall drop in radioactivity in this area of the gradient in the presence of guanidine probably reflects a decreased synthesis of RI. This is supported by the lack of buildup of large RI molecules (fractions 5 to 15) which would be expected if the release of mature RNA chains were inhibited.

The data in this report show that cytoplasmic extracts from poliovirus-infected cells synthesized a high level of 35*s* viral RNA over an extended period of time. During the first 7 min of incubation, predominately RI and dsRNA were produced (Fig. 3), but during a 90-min incubation period, the major product was 35*s* RNA (Fig. 2a). Guanidine inhibited the synthesis of 35*s* RNA, RI, and dsRNA in the in vitro reaction. The inhibition of synthesis of 35*s* RNA was moderately more pronounced than was the inhibition of synthesis of RI or dsRNA. This is analogous to results obtained during experiments conducted in vivo with the inhibitor (5). Because of uptake phenomena in intact cells, pool sizes of precursors, and electrolytes, it is not possible to accurately compare levels of RNA synthesis in vivo with those observed in vitro. Guanidine had no measurable effect upon elongation and termination, but apparently hindered RNA chain initiation. The data do not reveal the exact mechanism whereby guanidine blocks viral RNA synthesis, but suggest that extracts that are capable of initiating the synthesis of polynucleotide chains and producing 35*s* viral RNA are susceptible to the inhibitory effects of guanidine in vitro. Others have postulated that guanidine interferes with the assembly of precursor molecules during the formation of functional polymerase from a precursor pool (22, 27). If this were the case, then guanidine should not directly interfere with RNA chain elongation or termination.

During the past 4 years, the 5′ end of poliovirus RNA has been shown to contain a small covalently linked protein designated VPg (genome-linked virus protein [1, 19, 24]). Since the initial discovery other picornaviruses, such as foot-and-mouth disease virus, encephalomyocarditis virus, and rhinovirus, have been demonstrated to contain similar proteins (see reference 28 for a review). Because VPg is found on nascent strands of RI of poliovirus, it has been suggested that it acts as a primer for RNA synthesis (24) and possibly serves a role during the maturaton of virions. Whether this unique protein is essential for guanidine sensitivity of virus remains to be determined. However, both genetic (7) and biochemical (8, 17) studies suggest that capsid proteins are determinants of guanidine sensitivity and resistance of poliovirus. Perhaps the synthesis and encapsidation of viral RNA are closely regulated processes that require both capsid subunits (or their precursor) and VPg. The interaction of guanidine with capsid peptides or VPg could cause multiple aberrations during viral growth, such as blockage of RNA synthesis and blockage of maturation, and prevent capsid proteins from associating with smooth membranes.

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