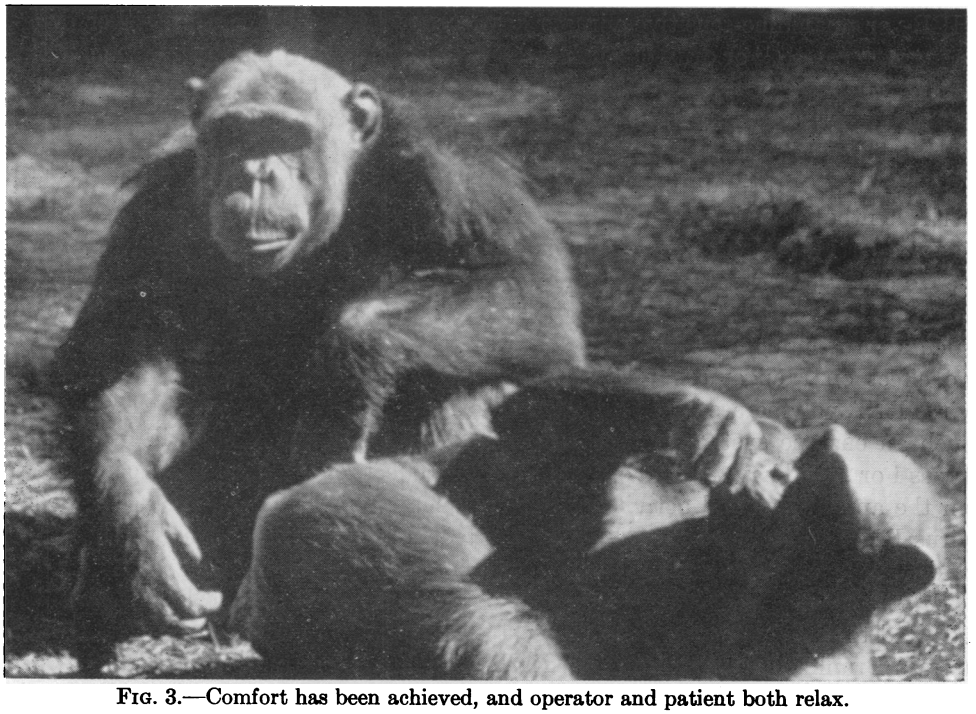

FIG. 3.—Comfort has been achieved, and operator and patient both relax.

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*POLIOVIRUS-INDUCED RNA POLYMERASE AND THE EFFECTS OF VIRUS-SPECIFIC INHIBITORS ON ITS PRODUCTION**

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Communicated by Frank L. Horsfall, Jr., April 19, 1963

In the last few years new experimental approaches have been found to the study of virus-specific biosynthesis in animal virus reproduction. This report describes recent results obtained in the study of the mechanism of action of two virus-specific inhibitors,[1–16] and provides evidence which bears on the virus-specific nature of the virus-induced RNA polymerase.[17–20]

2-(α-Hydroxybenzyl)-benzimidazole (HBB)[1–5] and guanidine[4, 10–16] inhibit completely and specifically the multiplication of many small, RNA-containing, lipid-free animal viruses (picornaviruses[21]). These compounds have no effect or only minor effects on the multiplication of viruses which belong to other major groups.[1, 2, 10] Similarly, the metabolic activities and growth rate of host cells are unaffected or only minimally affected by HBB[1, 3] or guanidine.[12, 13, 16] Mutants of

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HBB- or guanidine-sensitive viruses have been obtained which are either drug-resistant[1, 4, 5, 11, 14] or drug-dependent.[6, 7, 14, 22] It is known that HBB prevents the synthesis of the viral RNA of drug-sensitive viruses,[3, 8] but is required for replication of the RNA of drug-dependent mutants.[9] There is some evidence that guanidine has similar effects.[15, 16]

Recently, a virus-induced polymerase which is thought to be responsible for viral RNA synthesis has been found in Mengovirus-infected cells.[17–20] The enzyme has the properties of an RNA-dependent RNA polymerase, although dependence has yet to be directly demonstrated. The finding of a new RNA polymerase in cells infected with a picornavirus suggested a study of the effects of HBB and guanidine on such a virus-induced RNA-synthesizing system. Since HBB and guanidine have no effect on Mengovirus multiplication, it was necessary first to demonstrate virus RNA polymerase in cells infected with a drug-sensitive virus. We have found such an enzyme system in the cytoplasm of HeLa cells infected with poliovirus types 1 or 2, and have carried out a study of the effects of the virus-specific inhibitors on the appearance and activity of this enzyme system. The appearance of poliovirus RNA polymerase was also investigated in cells infected with drug-resistant or drug-dependent mutants.

Materials and Methods.—Viruses: Poliovirus type 2 (strain P 712-Ch-2ab), adapted to HeLa cells, was used in most experiments; in some, poliovirus type 1 (strain Brunhilde) and two of its variants were used; one was resistant to 100 μg guanidine per ml, and the other was dependent on guanidine. The dependent variant was grown and titrated in the presence of 100 μg/ml of guanidine. Poliovirus type 1 strains were kindly made available by Dr. Nada Ledinko, The Public Health Research Laboratories of the City of New York.

Virus RNA polymerase: HeLa cells were grown in suspension in 150 ml volumes of Eagle's spinner medium[23] with 4 mM glutamine and 10% calf serum. When the number reached 5 to 6 $\times$ 10^5 cells/ml, the cells were harvested by centrifugation and redispersed in 2 ml of undiluted poliovirus stock at a multiplicity of 5 to 10. After a 0.25-hr adsorption period at room temperature, 150 ml of warm medium without serum was added and incubation at 37°C begun (zero time). After the appropriate length of time, cells were harvested by centrifugation, dispersed in 0.25 *M* sucrose containing 0.001 *M* $MgCl_2$ (sucrose-Mg), recentrifuged, and either used directly, or frozen as a pellet. In most experiments (exceptions are noted) cells were homogenized in an all glass homogenizer after addition of 20–40 volumes of ice-cold water. All further operations were carried out at 0–4°C. The cell homogenate was adjusted to a final concentration of 0.1 *M* Tris-HCl buffer (pH 7.6), 0.005 *M* $MgCl_2$, and 0.5 *M* NaCl, and after 5–10 min, the viscous suspension was diluted 2-fold with water to reduce viscosity, and the nuclei, whole cells, and released nucleoprotein removed by centrifugation at 600 $\times$ *g* for 10 min. The suspension was then centrifuged at 78,000 $\times$ *g* for 1.5–2.0 hr, and the resulting pellet dispersed in sucrose-Mg at 2.5–10 mg of protein/ml. Details of this method will be published.[19] Such a preparation, which could be stored at −20°C for at least one week with retention of 75% or more of the initial activity, will be referred to as an enzyme preparation. Assay conditions were identical to those used for the Mengovirus polymerase.[17–19] The C^{14}-nucleoside triphosphates used were purchased from Schwarz BioResearch, Inc.

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TABLE 1

VIRUS RNA POLYMERASE ACTIVITY IN HELA CELLS INFECTED WITH POLIOVIRUS 2

	cpm/mg protein	
	Infected	Uninfected
Complete (GTP-C^{14})	155	4
-ATP, -UTP, -CTP	5	..
+ 5 mM Mn^{++}	32	..
Complete (ATP-C^{14})	123	36
-UTP, -GTP, -CTP	47	34
Complete (UTP-C^{14})	322	85

Infected cells were harvested 5 hr after infection with poliovirus type 2, suspended in sucrose-Mg and homogenized in a VirTis homogenizer.[24] The nuclei (600 × *g*, 8 min) and mitochondria (10,000 × *g*, 10 min) were discarded and the microsomal fraction (105,000 × *g*, 1 hr) was resuspended in sucrose-Mg at about 5 mg/ml. The assay mixture (0.5 ml) contained 1 μg actinomycin, 20 μg phosphoenolpyruvate kinase, 5 μmoles phosphoenolpyruvate, 5 μmoles magnesium acetate, 30 μmoles Tris-HCl buffer (pH 8.1) and 0.2 ml of the microsomal suspension. Either 73 mμmoles of GTP-C^{14} (2,100 cpm/mμmole), 96 mμmoles of ATP-C^{14} (690 cpm/mμmole), or 25 mμmoles of UTP-C^{14} (7,250 cpm/mμmole) was added to each tube along with approximately equal amounts of the other nucleoside triphosphates which were unlabeled. After 15 min incubation at 37° C, the reaction mixture was chilled in ice and 0.5 ml of cold 0.1 *M* sodium pyrophosphate added,[25] followed by 5 ml of cold 0.5 *M* perchloric acid (PCA). After 10 min in ice, the tubes were centrifuged in the cold and the resulting precipitate washed 3 times with PCA. After one wash in ethanol-ether (1:1) the precipitate was dissolved in 2 ml of concentrated formic acid, dried onto aluminum planchets, and counted in a windowless gas-flow counter. Protein was determined by the method of Lowry *et al.*[26] All results were corrected by subtraction of the counting rate of an unincubated sample.

Results.—Virus RNA polymerase in cells infected with poliovirus 2: As is shown in Table 1, microsomal preparations from poliovirus-infected HeLa cells incorporated forty times more GTP-C^{14} than did similar preparations from uninfected cells. Omission of unlabeled triphosphates markedly depressed incorporation, and the enzymatic activity was inhibited by manganese ions, as has been demonstrated for the Mengovirus RNA polymerase.[17–20] With C^{14}-labeled ATP and UTP, the microsomal preparations from infected cells showed 3–4 times greater incorporating activity than those from control cells.

The kinetics of virus RNA polymerase appearance in poliovirus-infected HeLa cells are shown in Figure 1. The polymerase activity did not appear until after 2 hr of infection, rose to a maximum at 4 hr, and then declined. The decrease was variable from experiment to experiment, and the apparent rise between 5 and 6 hr shown in Figure 1 is due to variation. The appearance of virus polymerase correlates well with the increase in virus (Fig. 1) and infectious viral RNA.[28] However, the results suggest that maximal amounts of virus RNA polymerase are made before the full yields of virus RNA and virus particles are produced.

Inhibition by guanidine and HBB of virus RNA polymerase appearance in infected cells: Guanidine had no effect on the activity of virus RNA polymerase preparations *in vitro* (Table 2). The lack of effect was evident at pH 8.1, the usual pH for assay, as well as at pH 7.5 or 7.2. HBB also had no effect on incorporation of GTP-C^{14} into an acid insoluble product in the cell-free system.

In contrast to the lack of direct effect on the polymerase, both compounds prevented the appearance of the virus RNA polymerase activity in HeLa cells infected with poliovirus. As is shown in Table 3, the microsome fraction from cells infected in the presence of HBB or guanidine incorporated no more GTP-C^{14} than did the microsome fraction from uninfected control cells.

It was next of interest to determine the effect of guanidine and HBB when added during the exponential increase phase in virus multiplication. Guanidine or HBB was added at 3 hr postinfection when 50 per cent of the enzyme activity had already appeared in the infected cells. After addition of either compound, there was not only no further increase in enzyme, but in fact a marked decrease occurred by

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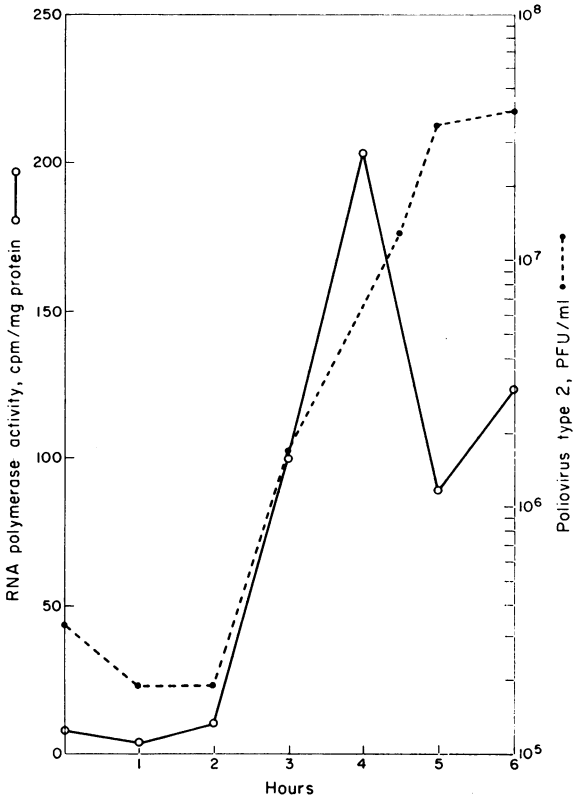

FIG. 1.—Specific activity of virus RNA polymerase after infection of HeLa cells with poliovirus type 2; growth curve of virus. Enzyme preparations were made from infected cells which had been incubated for various periods. Activity was assayed as described in Table 1, with 32 mμmoles of GTP-C^{14} (2,100 cpm/mμmole) as precursor. Results are expressed as cpm/mg/protein.

Plaque assays of virus were carried out in primary monkey kidney cell cultures.[27]

TABLE 2

EFFECT OF GUANIDINE AND HBB ON THE ACTIVITY OF THE VIRUS RNA POLYMERASE

	cpm
Complete	155
-ATP, -CTP, -UTP	5
+ 0.1 *M* guanidine	171
Complete (pH 7.5)	112
+ 0.1 *M* guanidine	101
Complete	568
+0.15 mM HBB	585

The conditions of experiments with guanidine were identical to those described in Table 1. GTP-C^{14} was used as the labeled nucleotide. The experiment with HBB shows higher incorporation values since the samples were collected at 4.25 rather than 5 hr, the enzyme preparation employed was made by water homogenization (cf. *Materials and Methods*) and 32 mμmoles of GTP-C^{14} (5,080 cpm/mμmole) was added to each reaction mixture.

4.25 hr (Table 4). Thus, the enzyme already formed appeared to have a short life. That the inactivation was not due to a direct effect of HBB or guanidine on the enzyme system is suggested by the fact that addition of the compounds to the cell-free GTP-C^{14}-incorporating system had no effect on its activity (Table 2).

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TABLE 3

INHIBITION BY GUANIDINE AND HBB OF VIRUS RNA POLYMERASE APPEARANCE IN HELA CELLS INFECTED WITH POLIOVIRUS 2

Compound present	cpm/mg protein
None	209
Guanidine, 1 mM	12
None	582
HBB, 0.2 mM	12

After infection, cells were resuspended in medium containing guanidine or HBB. Enzyme was prepared at 5 hr postinfection for the guanidine experiment and 4.25 hr for the HBB experiment, and assayed by the procedure described in Table 1. 73 mμmoles of GTP-C^{14} (2,100 cpm/mμmole) was used for the guanidine experiment and 32 mμmoles of GTP-C^{14} (5,080 cpm/mμmole) for the HBB experiment.

TABLE 4

INHIBITION BY GUANIDINE AND HBB OF VIRUS RNA POLYMERASE APPEARANCE IN HELA CELLS INFECTED WITH POLIOVIRUS 2

Compound added	Time of harvest, hr	cpm/mg protein
None	3	252
None	4.25	582
Guanidine at 3 hr	4.25	7
HBB at 3 hr	4.25	64

1 mM guanidine or 0.2 mM HBB was added to infected cells 3 hr after the virus adsorption period. Enzyme preparations were assayed as described in Table 1, with 32 mμmoles of GTP-C^{14} (5,080 cpm/mμmoles) as precursor.

Virus RNA polymerase in cells infected with drug-resistant or drug-dependent mutants of poliovirus 1; effects of guanidine: To obtain additional evidence as to the virus-specific nature of the RNA polymerase which appears in the cytoplasm of poliovirus-infected cells, experiments were undertaken with drug-resistant and drug-dependent mutants. The drug-resistant mutant of poliovirus 1 multiplied to high yields in the presence of 1 mM guanidine; the dependent mutant required guanidine for replication. Experiments were carried out to determine whether the resistant mutant could initiate enzyme production in spite of the presence of guanidine, and whether guanidine was required for enzyme production by the dependent mutant. The results of these experiments are summarized in Table 5. As can be seen, virus RNA polymerase was not demonstrable in cells which had been infected with the drug-sensitive wild type of poliovirus 1 and treated with guanidine. Enzyme activity was demonstrable in cells infected with drug-resistant virus regardless of whether guanidine was present in the growth medium. While the activity in untreated cells infected with the resistant mutant was significantly lower than that in such cells infected with the sensitive wild type, the yields of infectious particles were also lower. Finally, enzyme activity developed in cells infected with the drug-

TABLE 5

EFFECT OF GUANIDINE ON THE APPEARANCE OF THE VIRUS RNA POLYMERASE IN HELA CELLS INFECTED WITH DRUG-RESISTANT OR DRUG-DEPENDENT POLIOVIRUS MUTANTS

Poliovirus 1	Guanidine, 1 mM, present during infection	cpm/mg protein
Sensitive (wild type)	−	480
"	+	3
Resistant mutant	−	206
"	+	362
Dependent mutant	−	3
"	+	472

Cells were infected with various cloned mutant strains of poliovirus type 1 and infection allowed to proceed for 4 hr in the presence or absence of 1.0 mM guanidine. Enzyme preparations were assayed as described in Table 1, with 32 mμmoles of GTP-C^{14} (5,080 cpm/mμmole) as precursor.

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dependent virus only when the cells were incubated in the presence of guanidine.

It should be emphasized that virus RNA polymerase, produced in cells infected with drug-dependent virus in the presence of guanidine, did not require the compound for activity in the cell-free system. The virus RNA polymerase from such cells was sensitive to manganese, as was the enzyme produced in guanidine-treated cells infected with resistant virus.

Discussion.—From the foregoing results, it appears that poliovirus, like Mengovirus, causes the appearance in infected cell cultures of an RNA polymerase activity which is either not present in the uninfected cells, or is present at less than 2 per cent of the maximal level in the infected culture. The time course of enzyme appearance, and the correlation of activity with the growth of guanidine-sensitive, -resistant, or -dependent virus strains implicates the enzyme in the process of viral RNA synthesis.

As to the mechanism of action of guanidine and HBB, there is reason to believe that they specifically prevent virus-induced protein synthesis. Biochemical studies on cells infected with bacteriophage or DNA animal viruses have provided evidence that new enzyme activities and new protein antigens appear in cells after infection.[29, 30] In the case of bacteriophage infection a new class of messenger RNA's made soon after infection appear to determine the specificities of the new proteins.[31] As for the reproduction of DNA animal viruses, it is known that synthesis of RNA is a necessary prerequisite.[32, 33]

Mengovirus, a small lipid-free animal virus (picornavirus), causes, within an hour after infection, inhibition of cellular RNA and protein synthesis by separate processes which are inhibitable by puromycin and fluorophenylalanine and which therefore involve protein synthesis.[34] Poliovirus has similar effects.[35] Mengovirus and poliovirus also cause the synthesis of a new RNA polymerase and of coat protein. There is reason to believe that viral RNA or a copy of viral RNA supplies the information for the synthesis of these new proteins, i.e., two presumed inhibitors of cellular macromolecular synthesis, the polymerase, and the coat protein.[20, 34, 36] The observations that the RNA's of f2 bacteriophage[37] and of poliovirus[38] can *in vitro* determine the specificity of their respective coat proteins further strengthens the concept of viral RNA acting as a messenger RNA.

Both HBB and guanidine suppress the production of virus RNA polymerase in cells infected with drug-sensitive virus and also prevent the synthesis of virus RNA and coat protein. Furthermore, it has been found that guanidine can at least partially prevent the virus-induced depression of cellular RNA synthesis,[16, 39] and that both guanidine[10] and HBB[1] markedly palliate viral cytopathic effects. These facts, taken in conjunction with the concept that the viral RNA acts as a messenger RNA to provide the information for the synthesis of the new proteins, suggest that HBB and guanidine can interfere with the messenger function of viral RNA. In the case of the drug-dependent mutants, it would appear that the compound is required for the messenger function of viral RNA to be manifest. Since many of the details of the infective process are yet to be worked out, a more general form of this hypothesis may be useful, namely, that these compounds inhibit most if not all virus-induced protein synthesis. In any event, the effects of HBB and guanidine are reversible: no permanent damage to the viral RNA results from treatment of infected cells with these compounds.[3, 12]

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* Aided by a grant from The National Foundation.

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