Note added in proof: There has recently appeared an abstract of a paper on the same subject as the present paper [de Boor, C., and R. E. Lynch, *Notices Amer. Math. Soc.*, **11**, 681 (1964)]. An abstract of the present paper also appeared *loc. cit.*, p. 680.

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*RESCUE OF DRUG-REQUIRING AND DRUG-INHIBITED ENTEROVIRUSES**

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The RNA of small lipid-free animal viruses (picornaviruses) directs the synthesis of an enzyme which is responsible for the replication of the viral RNA in the infected cell.[1, 2] The synthesis of the virus-directed RNA polymerase can be specifically inhibited with guanidine hydrochloride and 2-(α-hydroxybenzyl)-benzimidazole (HBB).[2] In drug-treated cells neither viral RNA nor complete infectious virus is produced.[3–6] These facts, as well as the availability of drug-resistant [7–9] and drug-dependent [6, 10–12] mutants of picornaviruses, made it possible to explore the question of whether the RNA polymerase synthesized under the genetic control of one virus is capable of replicating the RNA of another virus in the same cell.

Two basic experimental designs were used. We studied the multiplication of drug-dependent viruses in the *absence* of the drug in cells simultaneously infected

with an assisting, drug-independent virus. The drug-independent viruses used were either drug-sensitive or drug-resistant. Conversely, we also studied the multiplication of drug-sensitive viruses in the *presence* of the drug in cells simultaneously infected with an assisting, drug-resistant or drug-dependent virus. Multiplication of drug-dependent or drug-inhibited viruses with the aid of an assisting virus was termed "virus rescue" or, for short, "rescue." The multiplication of complete, rescued virus was taken as an indication of synthesis of its RNA by the RNA polymerase of the assisting virus. Sometimes an increase in infective RNA of the rescued virus was measured directly.

It will be shown that either guanidine-sensitive or guanidine-resistant poliovirus, replicating in HeLa cells, permits the simultaneous multiplication of guanidine-dependent mutants of poliovirus in the absence of the drug. Conversely, guanidine-dependent or guanidine-resistant poliovirus can rescue guanidine-sensitive poliovirus strains in HeLa cells treated with guanidine. The genotype of the rescued virus is that of the parent virus, but phenotypically the rescued virus has acquired the capsid of the assisting virus. Complementation tests with three pairs of guanidine-dependent poliovirus strains have so far yielded no evidence of rescue. We have also found that drug-sensitive ECHO 7 virus rescues HBB-dependent Coxsackie A9 virus in monkey kidney cells.

Some of the results reported here have been independently obtained by Cords and Holland,[13, 14] by Wecker and Lederhilger,[15] and by Agol and Shirman.[16]

Materials and Methods.—Viruses: (1) Guanidine-sensitive polioviruses: type 1 (Brunhilde) and type 2 (P712-ch-2ab).[2] (2) Guanidine-resistant polioviruses: type 1 (Brunhilde) and type 2 (P712-ch-2ab). These strains were derived from the sensitive strains by passage in the presence of 1 mM guanidine and subsequent plaque purification. They were completely resistant to 1 mM guanidine. (3) Guanidine-dependent polioviruses: type 1 strains of Ledinko,[2, 12] Loddo,[10] and Lwoff.[17] The Loddo strain (obtained through the courtesy of Dr. B. Loddo) was derived from poliovirus 1, strain Brunenders, and had undergone 123 passages in the presence of guanidine. It was plaque-purified in this laboratory. The Lwoff strain, originally derived from a guanidine-dependent variant of Dr. B. Loddo, was kindly made available by Drs. A. Lwoff and M. D. Scharff. The guanidine-dependent strains were grown in the presence of 1 mM guanidine. All polioviruses used in this study were propagated in HeLa cells. (4) HBB-sensitive ECHO 7 virus was the prototype strain Wallace.[7] (5) HBB-dependent Coxsackie A9 (Woods) virus was isolated in this laboratory and grown in the presence of 0.1 mM HBB.[11] ECHO 7 and Coxsackie A9 viruses were propagated in primary rhesus monkey kidney cells.

Cells: HeLa cells, obtained through the courtesy of Dr. B. Mandel, were grown in monolayer cultures on 60-mm plastic Petri dishes in Eagle's minimum essential medium[18] supplemented with 10% calf serum. Primary rhesus monkey kidney cultures were prepared as described before.[7]

Immune sera: Type-specific immune sera against poliovirus 1 (Brunhilde) and poliovirus 2 (P712-ch-2ab) were prepared in this laboratory by hyperimmunization of rabbits. For some experiments poliovirus 1 (Brunhilde) monkey antiserum, provided by The National Foundation, or poliovirus 2 rabbit antiserum (purchased from Microbiological Associates, Inc.) was used. ECHO 7 (Wallace) rabbit antiserum was purchased from Microbiological Associates, Inc. Coxsackie A9 (Woods) antiserum was prepared by hyperimmunization of rabbits in this laboratory.

Chemicals: DL-2-(α-hydroxybenzyl)-benzimidazole and recrystallized guanidine·HCl were obtained through the kindness of Dr. A. F. Wagner of Merck, Sharp and Dohme Research Laboratories, Rahway, New Jersey.

Virus assay: The plaque assay was used throughout. A previously described procedure[3] was used with minor modifications. For HeLa cells, the overlay medium contained 5% calf serum, instead of the 2% used for monkey kidney cells. Guanidine-dependent poliovirus was assayed in the presence of 1 mM guanidine, and HBB-dependent Coxsackie A9 virus in the presence of 0.1 mM HBB.

TABLE 1

(*A*) RESCUE OF GUANIDINE-DEPENDENT TYPE 1 POLIOVIRUS BY GUANIDINE-SENSITIVE TYPE 2 POLIOVIRUS

Viruses Inoculated		Hr after virus inoculation	Yield (PFU) per Culture	
Rescued virus	Assisting virus		Rescued virus (dependent)	Assisting virus (sensitive)
No Guanidine in the Medium				
G.-dep. type 1	—	3	5.0×10^4	—
		10	1.5×10^5	—
G.-dep. type 1	G.-sens. type 2	3	7.5×10^5	
		10	3.9×10^7	9.5×10^8
—	G.-sens. type 2	3	—	
		10	—	8.0×10^8

(*B*) RESCUE OF GUANIDINE-SENSITIVE TYPE 2 POLIOVIRUS BY GUANIDINE-DEPENDENT TYPE 1 POLIOVIRUS

Viruses Inoculated		Hr after virus inoculation	Yield (PFU) per Culture	
Rescued virus	Assisting virus		Rescued virus (sensitive)	Assisting virus
Guanidine in the Medium				
G.-sens. type 2	—	3	2.3×10^4	—
		10	2.0×10^4	—
G.-sens. type 2	G.-dep. type 1	3	5.0×10^4	7.0×10^4
		10	1.2×10^7	1.0×10^9
—	G.-dep. type 1	3	—	4.5×10^5
		10	—	4.0×10^9

The experiment was carried out in a 37°C constant temperature room. 2×10^6 HeLa cells, in a 60-mm plastic Petri dish, were inoculated with 0.5 ml of a mixture of equal parts of guanidine-dependent poliovirus 1 (Loddo) and guanidine-sensitive poliovirus 2. The input multiplicity of each virus was 50–100 PFU per cell. In the control experiments, the HeLa cells were infected with either virus alone. After 30 min adsorption, the infected cells were washed 3 times with phosphate-buffered saline (PBS),[19] and then maintained for additional 30 min in the presence of appropriate antisera to inactivate unadsorbed virus. After removal of antisera by 2 washings with PBS, 5 ml of Eagle's medium without (*A*) or with 1 mM guanidine (*B*) was added to the cultures.

At the time of harvest, infected cells and culture fluids were frozen and thawed 3 times. The guanidine-dependent virus was assayed in the presence of 1 mM guanidine, and the drug-sensitive virus in the absence of the compound. Five hr after the first overlay, a second overlay containing appropriate antiserum was added (type 2 antiserum for type 1 virus assay, and type 1 antiserum for type 2 virus assay), to suppress the appearance of plaques of the heterologous virus.

Results.—Rescue of guanidine-dependent and guanidine-sensitive polioviruses: Table 1*A* shows the results of an experiment in which HeLa cells were doubly infected with guanidine-dependent poliovirus type 1 (strain Loddo), and guanidine-sensitive poliovirus type 2, in the absence of compound. It can be seen that at 10 hr the doubly infected, untreated culture yielded 3.9×10^7 PFU of poliovirus 1, whereas the untreated culture singly infected with dependent virus yielded only 1.5×10^5 PFU. Thus, in the presence of an assisting virus, the dependent virus was able to multiply without guanidine. The yield of the rescued virus was about 1 per cent of that obtained when dependent virus was grown under optimal conditions in the presence of guanidine (Table 1*B*). Similar results were obtained with the drug-dependent strains Ledinko and Lwoff.

The rescued type 1 virus shared the following properties with the drug-dependent parent virus: (1) It was genotypically type 1 virus as shown by the fact that its progeny was neutralized by type 1 but not by type 2 antiserum (see below). (2) It was guanidine-dependent. This was shown by examining 75 plaques (clones) of rescued type 1 virus from this and other, similar experiments. All clones consisted of guanidine-dependent virus populations, with back mutation indices from guanidine dependence to independence characteristic for each of the guanidine-dependent strains employed, i.e., 10^{-4} for strain Loddo, and approximately 10^{-3} for strains Ledinko and Lwoff.

Table 1*A* also shows that multiplication of the rescued guanidine-dependent virus was already in progress 3 hr after virus inoculation.

A very limited but significant increase of the type 1 virus occurred also in the *singly infected* culture, although no guanidine was present (Table 1*A*). This increase was shown to be due to the multiplication of drug-independent back mutants present in the inoculum of drug-dependent virus. Similar results have previously been obtained with enterovirus mutants requiring HBB.[11]

Table 1*B* shows the results of the converse experiment in which guanidine-treated HeLa cells were doubly infected with guanidine-sensitive poliovirus 2 and guanidine-dependent poliovirus 1 (Loddo). Without the assisting virus, the drug-sensitive type 2 virus did not multiply in guanidine-treated cells. However, when guanidine-dependent type 1 virus was replicating in the same culture, guanidine-sensitive virus did multiply, yielding about 1 per cent of the amount obtained in the absence of guanidine (Table 1*A*). The rescued virus was genotypically type 2 virus (see below), and it was as sensitive to guanidine as the parent virus.

To summarize, the data presented in Table 1 demonstrate that guanidine-dependent virus can multiply in the absence of the compound, and that guanidine-sensitive virus can do so in the presence of the compound, provided that a suitable assisting virus is concurrently multiplying in the same culture.

Table 2 shows the results of experiments in which rescue of the infective RNA by assisting virus was demonstrated directly. Part *A* of Table 2 illustrates rescue of the infectious RNA of guanidine-dependent virus by type 2 assisting virus. Part *B* of Table 2 shows that in the converse experiment, the assisting type 1 virus made possible a substantial increase in type 2 viral RNA, but a proportionate increase in complete virus did not occur, resulting in a comparatively high ratio (10^{-2}) of the titer of viral RNA to complete virus. One possible reason for this result may be that guanidine perhaps affects not only viral RNA polymerase synthesis but also some other process in the reproduction of enteroviruses, e.g., virus

TABLE 2

(*A*) RESCUE OF INFECTIVE RNA OF GUANIDINE-DEPENDENT TYPE 1 POLIOVIRUS BY GUANIDINE-SENSITIVE TYPE 2 POLIOVIRUS

Viruses Inoculated		Hr after virus inoculation	Yield (PFU) per Culture — Rescued (dependent)	
Rescued virus	Assisting virus		RNA	Virus
No Guanidine in the Medium				
G.-dep. type 1	—	1¾	1.5×10^3	3.8×10^4
		7	5.0×10^3	3.2×10^5
G.-dep. type 1	G.-sens. type 2	1¾	5.0×10^3	2.9×10^4
		7	3.6×10^4	6.0×10^7

(*B*) RESCUE OF INFECTIVE RNA OF GUANIDINE-SENSITIVE TYPE 2 POLIOVIRUS BY GUANIDINE-DEPENDENT TYPE 1 POLIOVIRUS

Viruses Inoculated		Hr after virus inoculation	Yield (PFU) per Culture — Rescued (sensitive)	
Rescued virus	Assisting virus		RNA	Virus
Guanidine in the Medium				
G.-sens. type 2	—	1¾	1.9×10^3	1.5×10^4
		7	1.6×10^3	1.1×10^4
G.-sens. type 2	G.-dep. type 1	1¾	1.8×10^3	1.9×10^4
		7	1.5×10^4	1.5×10^6

For details of the experimental procedure see Table 1. At the time of harvest, the medium was removed, and the cells suspended in 2 ml of 0.02 *M* phosphate buffer, containing 5×10^{-4} *M* Na_2 EDTA (disodium ethylenediamine tetra-acetate) and 10^{-2} *M* tris (tris(hydroxyethyl)aminomethane) (pH 7.4). The cells were frozen and thawed 3 times, and a part of the suspension was used for assay of complete virus (see Table 1); another part was used for extraction of infective RNA with cold phenol which had been saturated with 0.02 *M* phosphate buffer. The RNA preparation was diluted in 10^{-2} *M* tris (pH 7.4) with 2 *M* $MgSO_4$.[20] HeLa cells were exposed to 0.2 ml of diluted material for 10 min at room temperature. The inoculum was removed by suction, the monolayer washed once with PBS and overlaid as described in Table 1. Table 2 lists only cell-associated virus and RNA. At 7 hr the released virus amounted to less than 10% of the cell-associated virus.

maturation.[21] At any rate, in all experiments in which drug-sensitive virus was grown with an assisting virus in the presence of guanidine, the virus yield was 4 to 75 times lower than in the converse experiment, i.e., rescue of dependent virus by a drug-independent one.

It may also be seen from Table 2, parts *A* and *B*, that at $1^3/_4$ hr after virus inoculation, when virus multiplication had not yet begun, the ratio of infective viral RNA to infective virus was as high as 10^{-1}, probably reflecting the "eclipse" of infective virus.

The antigenic type of the rescued virus: Genotypically, the antigenic type of the rescued virus was that of the parent virus, although phenotypically it assumed—at least predominantly—the antigenic character of the assisting virus. The evidence is presented in Table 3.

TABLE 3

THE PHENOTYPIC AND GENOTYPIC ANTIGENIC TYPE OF RESCUED POLIOVIRUS

Treatment	(*A*) Guanidine-Dependent Poliovirus Type 1 Rescued by Guanidine-Sensitive Type 2 Poliovirus* PFU			(*B*) Guanidine-Sensitive Poliovirus Type 2 Rescued by Guanidine-Dependent Type 1 Poliovirus† PFU		
	No serum	Type 1 serum	Type 2 serum	No serum	Type 1 serum	Type 2 serum
Virus and antiserum mixed before virus inoculation (determination of phenotype)	126	122	0	128	0	100
Antiserum added to cultures 5 hr after virus inoculation (determination of genotype)	126	0	120	128	126	0

* An equal volume of rescued guanidine-dependent poliovirus (see Table 1*A*) containing about 100 PFU per 0.1 ml was mixed with either polio type 1 or type 2 antiserum, or with PBS. The mixtures were incubated for 45 min at room temperature (28–29°C), and 0.2 ml was inoculated per plate. After 1 hr adsorption at 36°C the inoculum was removed and the cells were overlaid with agar overlay medium containing 1 mM guanidine. The group of plates to which type-specific antisera were added 5–6 hr after virus adsorption were processed in a similar manner, but using virus-PBS mixtures as inoculum. 5–6 hr after virus inoculation the antisera were added in a second overlay.

† The virus to be tested was the rescued guanidine-sensitive poliovirus listed in Table 1*B*. The overlay medium contained no guanidine.

The rescued guanidine-dependent virus (Table 1*A*) was exposed to type-specific antisera before or 5 hr after virus inoculation (Table 3*A*). When mixed with antiserum before virus-cell interaction, the virus was neutralized completely by type 2 antiserum, but not at all by type 1 serum, indicating that the virus had a type 2 capsid. On the other hand, when the antisera were added 5 hr after virus inoculation, by which time new virus had been synthesized, the opposite was found: the progeny of the rescued virus was neutralized by type 1 and not by type 2 antiserum, i.e., the RNA of the rescued virus coded for a type 1 protein coat.

Essentially analogous results were obtained with the rescued guanidine-sensitive virus (Table 1*B* and 3*B*). Since the rescued type 2 virus was also slightly neutralized by type 2 serum when the rescued virus and antiserum were mixed before plating (Table 3*B*), it is possible that some particles of the rescued virus contained parts of type 2 capsid protein.

Rescue experiments using guanidine-resistant virus as assisting virus: A guanidine-resistant type 2 virus was able to rescue both a guanidine-dependent type 1 virus in the absence of guanidine, and a sensitive type 1 virus in the presence of the drug. However, not all combinations resulted in rescue, e.g., a guanidine-resistant type 1 virus (derived from the drug-sensitive Brunhilde strain) did not rescue our standard drug-sensitive type 2 virus in guanidine-treated cultures. As expected, a double

infection with drug-sensitive type 1 and type 2 strains in the presence of guanidine did not result in any virus multiplication.

Complementation tests with guanidine-dependent poliovirus strains: Since two guanidine-dependent poliovirus 1 strains of different origin were available (strains Loddo and Ledinko), complementation tests were attempted, including also the Lwoff strain which was originally derived from the Loddo strain. No combination of drug-dependent mutants (strains Loddo and Ledinko, Loddo and Lwoff, Ledinko and Lwoff) yielded measurable multiplication of drug-dependent virus. There was only some multiplication of drug-independent back mutants; the extent of multiplication was similar to that observed in singly infected cultures.

Rescue of HBB-dependent Coxsackie A9 virus: Experiments analogous to those performed with polioviruses and guanidine in HeLa cells were also carried out with HBB-dependent Coxsackie A9 virus and drug-sensitive ECHO 7 virus in monkey kidney cells, and rescue of the dependent virus was demonstrated in the absence of HBB. The rescued virus required HBB for growth. The yield of rescued HBB-dependent Coxsackie A9 virus was lower than that of rescued guanidine-dependent poliovirus. This result may be due to the fact that under the conditions of the experiment at most 50 per cent of the cells in the culture were initially infected with Coxsackie A9 virus. This is in contrast to the poliovirus-HeLa cell system, in which all of the cells are initially infected, as determined by the infectious center technique.

No significant rescue of HBB-sensitive ECHO 7 virus by HBB-dependent Coxsackie A9 was demonstrated in cultures treated with 0.1 mM HBB. This phenomenon may be due to inhibitory effects of HBB on virus maturation,[3, 21] which the assisting virus would not be expected to alter.

Discussion.—Rescue phenomena are well known for DNA bacterial viruses and have recently also been reported for RNA phages.[22] We have studied guanidine- or HBB-dependent enteroviruses which apparently fail to synthesize viral RNA polymerase in the absence of the drugs, and drug-sensitive enteroviruses which cannot make viral RNA polymerase in the presence of inhibitor. Guanidine-dependent poliovirus 1 strains were shown to be able to multiply without the compound if drug-sensitive poliovirus 2 was concurrently replicating in the same culture. Under these conditions the synthesis of infective RNA of guanidine-dependent poliovirus was also directly demonstrated. Cords and Holland[13] similarly reported rescue of infective RNA of guanidine-dependent poliovirus 1 by various assisting enteroviruses.

That the assisting and rescued virus begin to multiply simultaneously was strongly suggested by the fact that multiplication of the rescued virus was already in progress 3 hr after virus inoculation. It is well known that the assisting poliovirus 2 has a latent period of 2–2$^1/_2$ hr. Wecker and Lederhilger have presented detailed evidence on the kinetics of concurrent multiplication of rescued and assisting virus.[15]

In addition to guanidine-dependent mutants, HBB-dependent Coxsackie A9 virus was also shown to be rescued by drug-sensitive virus.

The genotype of the rescued virus was, in all cases, that of the parent virus in the two properties studied: drug dependence and antigenic type. Thus, exchange of genetic material did not appear to play a role in the phenomena observed.

In general, similar results were obtained when drug-sensitive viruses were rescued by drug-dependent mutants in the presence of guanidine, although the yield of rescued drug-sensitive virus was always lower than that of rescued drug-dependent virus, which may be due to an inhibitory effect of guanidine on processes other than synthesis of viral RNA polymerase, e.g., virus maturation.[21] The same reasoning may be used to explain the failure of rescue of drug-sensitive ECHO 7 virus by HBB-dependent Coxsackie A9 virus in the presence of HBB.

Although guanidine-resistant poliovirus was shown to be able to rescue both guanidine-dependent and guanidine-sensitive polioviruses, not all poliovirus combinations have resulted in virus rescue. Thus, a guanidine-sensitive poliovirus 2 was not rescued by a guanidine-resistant poliovirus in the presence of guanidine, although the same type 2 virus could be rescued by guanidine-dependent virus.

The results obtained support the hypothesis that the viral RNA polymerase induced by a heterologous virus makes possible the multiplication of drug-requiring or drug-inhibited viruses. That the rescued virus may also depend on other virus-specific processes directed by the assisting virus has not been excluded. For example, we have found that the drug is required in the reproduction of guanidine-dependent poliovirus 1 (Ledinko) within the first hour after virus inoculation,[23] whereas detectable synthesis of viral RNA polymerase does not begin until about 1.5–2 hr later. It is of course possible that the synthesis of viral RNA polymerase actually begins shortly after infection, but cannot be detected by the techniques used. However, we cannot exclude the possibility that during the early part of the latent period a drug-requiring reaction other than the formation of the active viral polymerase takes place. This reaction may be a preparatory step in the synthesis of the active polymerase or may be wholly unrelated to polymerase synthesis.

Doubly infected cultures yielded 25 or more times as much assisting virus as rescued virus. The predominance of assisting over rescued virus particles was also reflected in the finding that the bulk of the capsid protein of the rescued virus is provided by the assisting virus, as was also reported by Holland and Cords.[14] The low yield of rescued RNA and virus (about 1% of that obtained with the virus under optimum conditions) does not necessarily mean that virus-induced RNA polymerase is less efficient in the synthesis of heterologous enterovirus RNA. This phenomenon could be explained on the assumption that most of the virus-induced RNA polymerase is occupied by homologous RNA.

So far our complementation experiments with guanidine-dependent poliovirus strains have been unsuccessful. The simplest hypothesis to explain this negative result is that the drug-requiring process of the three strains used concerns the same virus-directed protein or proteins.

At least formally, there is an obvious resemblance between enterovirus rescue and the formation of infectious Rous sarcoma virus (RSV) with the aid of a "helper" leucosis virus.[24] In both instances an assisting virus makes possible the multiplication of another virus which cannot multiply on its own. But whereas the assisting enterovirus makes possible the replication of the *genetic material* of a heterologous virus, the "helper" leucosis virus provides the *viral coat*. More information is needed to decide whether the "helper" leucosis virus provides additional functions, or macromolecules, which may be needed in the production of complete infectious RSV.

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*ON COMPACTNESS OF MAPPINGS**

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1. *Introduction.*—A mapping $f:X \to Y$ is *compact* [sometimes called proper, propre (Fr.), eigentlich (Ger.)] provided the counterimage $f^{-1}(K)$ of every compact set K in Y is compact. Also f is *monotone* provided the inverse $f^{-1}(y)$ of every point $y \in Y$ is a continuum (compact and connected) or else is the empty set.

A topological space X is *peripherally compact* provided that for each $x \in X$ and each open set U in X containing x, there exists an open set V in X with $x \in V \subset U$ and such that the boundary $Fr(V)$ is a compact set. In general this is a weaker property than local compactness.

As just indicated, the boundary of an open set V is denoted by $Fr(V)$. Also a connected open set is called a *region;* and in case the space under consideration is metric, $V_r(M)$ will denote the *spherical neighborhood of the set M of radius r*, i.e., the set of all points x at distance $< r$ from the set M. The empty set is denoted by Φ. We shall need to use the generally known fact that *if X and Y are Hausdorff spaces, any closed mapping $f:X \to Y$ with compact point inverses is a compact mapping.*

To verify this we let K be any compact set in Y. We may suppose $K \subset f(X)$