# Specific Inhibition of Replication of Animal Viruses

## Chemical inhibitors are helping to elucidate virus-specific processes in virus reproduction.

I. Tamm and H. J. Eggers

The process of virus reproduction is so closely bound up with the metabolism of the virus-infected cell that its study has been hindered by inability to distinguish experimentally between the two. The main difficulty has been the lack of specific chemical markers which would make possible the study of the synthesis of virus nucleic acids and proteins without measuring or affecting host-cell synthesis. In the exceptional instance of T2, T4, and T6 bacteriophages, which contain an unusual nucleotide, a very beautiful and comprehensive picture has been obtained of the biochemical and enzymatic changes in the virus-infected cells (*1*).

In the last few years, however, chemical compounds have been found which can distinguish between virus-induced and cellular processes and thus the knowledge of virus-specific events in the reproduction of animal viruses has begun to advance more rapidly. The mechanisms of action of these virus-specific inhibitors bear directly on questions concerning virus-specific features of the structure, synthesis, and functions of virus nucleic acids and proteins.

Virus reproduction can occur only inside a living cell, since the virus is dependent on the cell both for energy and for the building blocks that make up its substance. Various subcellular structures serve specialized functions in the mechanism of virus reproduction. The virus particle consists of a protein shell (the capsid) and a core of nucleic acid (*2*). The nucleic acid may be either DNA or RNA; no virus has both. It is the nucleic acid which carries into host cells the genetic information needed to reproduce the virus. Once inside the cell, the genetic material of the virus proceeds to function in violation of the well-balanced control mechanisms previously operating in the cell, so that new virus substance is produced. This may occur to the detriment of, or in addition to, the ongoing production of cell substance.

There are differences in the functions of viral genetic material, depending on whether it is DNA or RNA, but in either case virus nucleic acid probably serves as a template in two separate reactions. Viral DNA acts as a template in the synthesis of virus-specific messenger RNA's (*3*). These RNA's then serve as templates for the manufacture of virus-specific proteins. Viral DNA also must function as a template in its own replication. Thus, the functions of viral DNA are fundamentally similar to those of the chromosomal DNA of the host cells.

Viral RNA functions as messenger RNA in the synthesis of virus-specific proteins (*4*). It shares this function with the messenger RNA of host cells. In addition, however, viral RNA must in some way serve as a template in its own replication. Viral RNA is unique in its capacity as genetic determinant; no other RNA appears to have this function. It is possible that the RNA of some viruses does not itself function as messenger but serves as a template in the synthesis of messenger RNA's.

The genetic material of a virus clearly has to possess the information for the synthesis of viral coat protein. However, for many years there was no evidence that animal-virus nucleic acid also contained information for the synthesis of virus-specific enzyme proteins which would function in virus biosynthesis. It was thought that the host cell provided all the necessary enzymes.

There is now evidence that the enzymes which are responsible for the synthesis of nucleic acids of animal viruses may, at least in some cases, be new, virus-controlled proteins (*5–7*). There is no evidence, however, that any new, virus-specific enzymes which function in the synthesis of virus protein are made in the infected cell.

The crucial fact for the study of these processes is that, in spite of the intimate relationship between virus and host cell, the nucleic acid and protein of a given virus, and also its precise structure, are specific for that virus and quite distinct from those of the host cell which produces them. This fact suggested that it might be possible to inhibit the synthesis of virus-induced nucleic acid and proteins in infected cells without at the same time interfering with the synthesis or function of host-cell nucleic acids or proteins (*8, 9*). The idea that it may be possible to specifically inhibit virus-controlled macromolecular synthesis is based on the concept that selective compounds can be found or designed which will recognize and combine with an appropriate virus-specific target. The target might be viral nucleic acid itself, a virus-specific enzyme, or some other virus-specific component in the infected cell which plays an essential role in the process of virus reproduction. The assumption is that, when combined with the inhibitory compound, the virus-specific macromolecule would be unable to function. Probably weak forces, such as hydrogen bonds, would be involved in the interaction between the inhibitor and the virus-specific macromolecule, and the complex would be expected to be a reversible one.

A second approach to selective inhibition is based on the idea that there may be quantitative differences in the biosynthetic mechanisms of the virus and the host cell (*9*) which may provide a basis for selective inhibition of virus multiplication by chemical compounds, such as structural analogs of metabolites (*10*) required by both virus and host cell. Such antimetabolites would interfere with the synthesis or utilization of small-molecular precur-

Dr. Tamm is associate professor and Dr. Eggers is assistant professor at the Rockefeller Institute, New York 21.

sors of nucleic acid or protein (*11*, *12*). If incorporated, a structural analog of a metabolite might cause lethal synthesis—that is, the production of inactive viral progeny (*13*).

One of the main problems in achieving specific chemical inhibition of the replication of animal viruses is that of finding specific inhibitors (*9*, *12*, *14*, *15*). The search for such inhibitors has been either essentially random or guided by enlightened empiricism; a number of successes have come through educated guesses as to what type of compound might possess selective virus-inhibiting activity. The initial findings have then been extended through study of related compounds. Recent developments in molecular biology give promise that at some future date it may be possible to design inhibitors which will predictably and specifically interfere with the synthesis of particular kinds of nucleic acids (*16*).

We have been especially interested in the specific inhibition of small lipid-free RNA viruses (picornaviruses) (*17*) by 2-($\alpha$-hydroxybenzyl)-benzimidazole (HBB) and guanidine (*18–23*). Here we describe selective inhibition of the synthesis of viral RNA and protein by these compounds (*24–28*) and provide evidence which suggests that this inhibition may be due to inhibition of the production of virus-induced RNA polymerase (*6*). We also report studies which show that the RNA of drug-dependent mutant virus requires HBB or guanidine for its replication (*26*); inability of the RNA of dependent virus to replicate in the absence of these compounds may be due to failure of synthesis of virus-induced RNA polymerase (*6*).

In addition, we discuss briefly some of the findings which have been obtained with isatin $\beta$-thiosemicarbazone (*29*, *30*) and halogenated 2′-deoxyuridines (*31–34*). The thiosemicarbazone has an inhibitory effect which is specific for one major group of DNA viruses, the poxviruses, whereas the deoxyuridines have selective effects on several groups of DNA viruses.

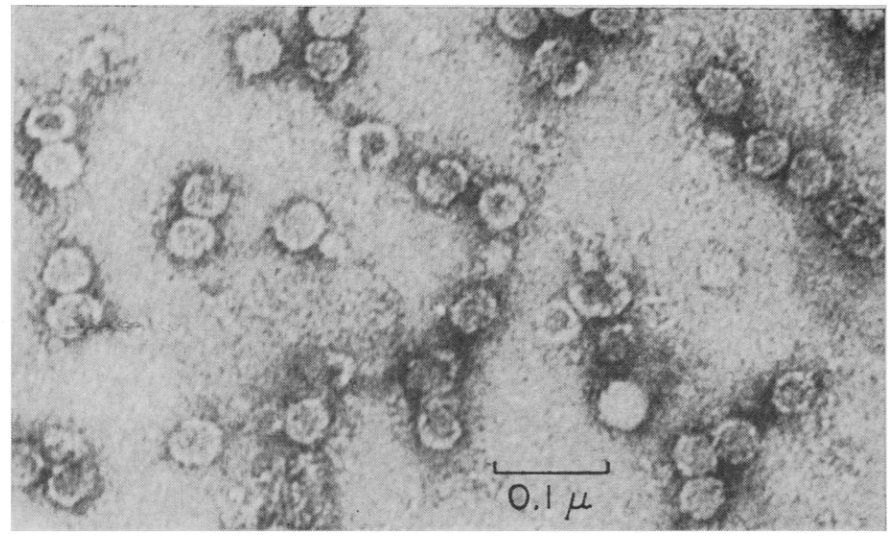



Fig. 1. ECHO 12 virus particles stained with uranyl acetate. Note the interior, which contains the RNA of the virus. The morphological units of the protein coat can be seen especially well on the few particles whose interior has not stained. [S. Halperen, Rockefeller Institute]

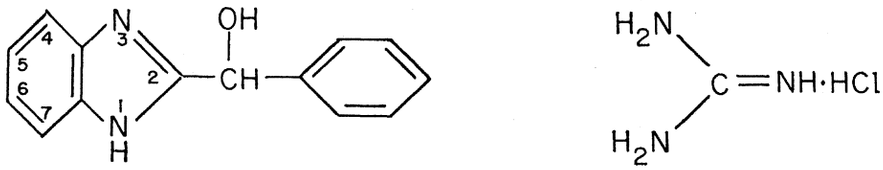
## The Picornaviruses

The small lipid-free RNA viruses or picornaviruses are of special interest for a number of reasons. As was pointed out earlier, the RNA of picornaviruses not only carries genetic information and functions as a template in its own replication but also serves as messenger RNA in the synthesis of virus-specific proteins. The RNA is the sole genetic material of the virus, and since the amount of RNA per virus particle is equivalent to only about $2 \times 10^6$ molecular-weight units (*35*), the genetic complexity of the small RNA viruses is severely limited (*36*).

The picornaviruses are about 27 to 30 millimicrons in diameter and contain RNA (about 25 percent) and protein (75 percent). The protein shell is made up of morphological units, the capsomeres, and has icosahedral symmetry (*37*). There may be about 60 capsomeres, each approximately 6 millimicrons in diameter. The mass of each must be equivalent to about 80,000 molecular-weight units. Since the molecular weight of the proteins in the coat of poliovirus is 25,000 (*38*), each capsomere may contain three protein molecules. The structure of these viruses is illustrated in Fig. 1, which shows ECHO type 12 virus particles stained positively with uranyl acetate.

The amount of RNA present in the virus particle is equivalent to about 6000 nucleotides. If a sequence of three nucleotides is necessary to code one amino acid, then it follows that the maximum number of amino acids that can be organized by the viral nucleic acid is 2000. Since the molecular weight of the protein subunits of poliovirus is 25,000 (*38*), each subunit is therefore made up of about 200 amino acids. Clearly, there is more than enough RNA in the virus to code for the protein of the capsid, even if more than one kind of protein is present, as suggested by electrophoretic heterogeneity of poliovirus protein (*38*). Furthermore, unless a significant part of the polyribonucleotide chain is genetically nonfunctional, there appears to be enough nucleic acid to code for several other proteins. There is some evidence that the nucleic acid of small RNA viruses may direct the production of perhaps four different proteins (*39*, *40*), or even more.

The first step in the interaction of any virus with a susceptible host cell is adsorption of the virus to the cell surface. In the attachment of virus to cell, specific groupings on the surface of the virus react with specific receptors on the surface of the cell (*41*, *42*). The main function of the protein coat of the virus is that of safely transmitting the genetic material of the virus from cell to cell. The receptors on the plasma membrane of the cell not only bind virus but also trigger an alteration in the capsid that results in profound changes in the biological properties of the virus particles, changes which culminate in an eclipse of the particles. This alteration in the capsid, triggered by the membrane receptor, may be the first stage in the release of viral nucleic acid from its very durable protein coat. The altered particle is probably passively taken into the cell (*43*), and the capsid may be digested by proteolytic enzymes of the cell (*41*).



2-(α-Hydroxybenzyl) benzimidazole (HBB)

Guanidine Hydrochloride

Fig. 2. Structures of two specific inhibitors of picornavirus reproduction.

For the genetic material of the virus this marks the beginning of a period of vigorous activity, but for the genetic material of the cell it is the beginning of a chain of events that ultimately lead to the destruction of the entire cell. At the time when the production of virus-induced RNA polymerase and of new virus nucleic acid and protein begins, or even before that time, there is already inhibition of the RNA-synthesizing system of the cell (*39*, *44*, *45*). Thus, cellular DNA is no longer able to make messenger RNA's. What is especially remarkable is that inhibition of the synthesis of cellular proteins occurs at the same time as, or even sooner than, inhibition of synthesis of cellular RNA. Therefore, separate virus-induced inhibitors have been postulated to account for inhibition of the synthesis of cellular protein and cellular RNA (*40*).

While these changes in cellular metabolism are taking place, a virus-induced RNA polymerase becomes detectable (*5*, *6*), and virus RNA and protein begin to appear at about the same time, followed a short time later by the completed virus particles (*24–26*, *46*). The virus-induced RNA polymerase is probably the enzyme system responsible for synthesis of viral RNA. The new, mature virus particles are released into the extracellular environment after a short delay. In natural infections, only a few cells are initially infected by the relatively few virus particles which succeed in entering the host and finding susceptible cells. The virus released from cells initially infected then attacks new cells. In studies of the mechanism of virus reproduction and of the effects of virus inhibitors, the culture is commonly inoculated with large amounts of virus, to assure nearly simultaneous infection of all the cells.

That the multiplication of small RNA viruses is not affected by actinomycin D (*47*) is of considerable interest. This antibiotic binds strongly to DNA (*48*) and inhibits synthesis of cellular RNA (*49*) by interfering with the template function of cellular DNA (*50*). The fact that actinomycin D does not inhibit the growth of small RNA viruses serves to differentiate replication of viral RNA from synthesis of cellular RNA. Experimentally, actinomycin D makes it possible to study virus-induced processes in cells in which cellular synthesis has been suppressed.

The virus-specific effects of HBB and guanidine on the biosynthesis of virus-induced macromolecules have opened an approach to the study of the specific mechanism involved in the replication of the genetic material of the picornaviruses. These compounds are also proving useful in the study of genetic fine structure in picornaviruses, variation in picornaviruses, and virus-induced changes in cell metabolism and structure. In short, the conditions are now particularly favorable for the study of the molecular and cellular biology of the small RNA viruses.

## Inhibition of Replication of Picornaviruses

Guanidine and HBB (Fig. 2) specifically inhibit the reproduction of many, though not all, picornaviruses (*18–23*, *27*, *51*). The specific virus-inhibiting activity of HBB was discovered as a result of extensive investigation of the relationship between the structure of benzimidazole derivatives and their virus-inhibiting activity (*8*, *9*, *12*, *14*, *52*, *53*). The virus-inhibiting activity of guanidine was detected in a chemotherapeutic screening system when the guanidine salt of hydroxyaminomethylene malononitrile was examined (*21*), and also when some simple guanidine derivatives were investigated to find whether the guanidine moiety might possess antiviral activity (*51*). Finally, it was inferred (*23*) that the inhibitory effect of 2-imino-5-methylhexahydro-*s*-triazine on the growth of poliovirus was attributable, not to the compound itself, but rather to one of its decomposition products—namely, guanidine—and experiments were carried out to test this inference.

Studies with benzimidazole derivatives were begun more than 10 years ago (*8*). The initial working hypothesis was quite simple: It was hoped that selective inhibition of nucleic acid synthesis might be obtained with benzimidazole derivatives which would not interfere directly with the synthesis or utilization of low-molecular-weight precursors of nucleic acids but would act, rather, on some regulatory mechanism that controls nucleic acid biosynthesis. The only experimental approach that seemed feasible was to use as inhibitors structural analogs of those vitamins known to play a role in nucleic acid biosynthesis. The vitamin $B_{12}$ area appeared attractive because it was new and thus no discouraging evidence was as yet available. Vitamin $B_{12}$ contains a benzimidazole nucleoside moiety, 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole (α-ribazole). In the studies with benzimidazole derivatives which have ensued, no results have thus far been obtained linking the virus-inhibiting activity of such derivatives with the function of vitamin $B_{12}$ in nucleic acid biosynthesis. However, all of the evidence is compatible with the idea that the virus-specific inhibitors HBB and guanidine prevent the expression of gene and messenger functions of viral RNA (*6*, *18*, *24*).

Through studies of structure-activity relationships several groups of benzimidazole derivates have been discovered, each characterized by special structural features and each possessing a striking, and different, biological activity. Many of the benzimidazole derivatives used in the early structure-activity studies have never been used again. However, from the results obtained with them, the conclusion was reached early that the virus-inhibiting activity and the toxicity of benzimidazoles can vary independently of each other (*9*, *54*). The idea of making further studies therefore seemed attractive, since it appeared that the biological activities of benzimidazoles could be modified in remarkable ways by alteration of their structure.

The first benzimidazole derivative

examined (*8*), 2,5-dimethylbenzimidazole, turned out to be neither highly active nor selective as an inhibitor of influenza virus replication (*54*). However, its reversible inhibitory effect on cellular processes made possible the first demonstration that a biosynthetic product of the host is implicated in virus interference (*55*). The critical finding was that 2,5-dimethylbenzimidazole prevented the establishment of virus interference by heat-killed influenza virus.

Certain structurally very different derivatives of benzimidazole are highly active inhibitors of cellular, and probably also of viral or virus-induced, RNA biosyntheses. 5,6-Dichloro-1-$\beta$-D-ribofuranosylbenzimidazole (DRB) inhibits incorporation of adenosine into cellular RNA (*56*, *57*). Structurally, DRB is an analog of purine nucleosides. For highest inhibitory activity, not only must the carbohydrate be ribose but there must be ribofuranose in the $\beta$ linkage (*58*). Inhibitory activity increases markedly with the number of halogen atoms substituted. DRB and related compounds have been helpful in the study of the role of RNA in protein synthesis in animal cells (*57*). With the aid of DRB it has also been possible to show that synthesis of RNA is a necessary step in the reproduction of DNA-containing animal viruses (*56*, *59*).

The first benzimidazole derivative to show virus-specific effects was 5-methyl-2-D-ribobenzimidazole (*60*). This compound and related derivatives are unique in that they *increase* the yield of certain strains of influenza virus from infected tissue without affecting the metabolic activities of the cells. The presence of the hydroxyl groups on the side chain appears to be essential for enhancing activity.

The virus-inhibiting action of HBB and guanidine is virus-specific; these compounds inhibit the reproduction of viruses belonging to a single major group and have no effects, or only minor effects, on host cells (*18*, *27*, *28*, *51*). The structural requirements for selective virus-inhibiting activity are stringent for both HBB and guanidine in that, in general, structural modifications reduce or eliminate virus-inhibiting activity (*14*, *27*, *51*, *53*). Guanidine and HBB are quite different with respect to molecular size, aromaticity, steric points, number of nitrogen atoms, and basicity. Yet, because of similarities in their action, it seems likely that these compounds have a feature in common which is essential for specific virus-inhibiting activity. The

$$>N-\overset{|}{C}=N-$$

sequence in both may be such a feature. Though this may be an essential feature, it is clear that, in HBB, this sequence is not of itself sufficient for biological activity, since 2-($\alpha$-hydroxybenzyl)-imidazole and 2-hydroxymethylbenzimidazole are both completely inactive. Thus, both the benzenoid ring and the phenyl radical are also important. There is much experimental evidence that substitution at the $\alpha$-carbon atom is also critically important.

It thus appears that the overall configuration of HBB, the

$$>N-\overset{|}{C}=N-$$

sequence, and substitution at the $\alpha$-carbon atom are all important aspects of the structure of HBB. O'Sullivan and Sadler (*61*) have suggested that the formation of intramolecular hydrogen bonds involving the hydroxyl group may be of importance for the selective virus-inhibiting activity of HBB. This is unlikely, since 2-($\alpha$-methoxybenzyl)-benzimidazole, a compound in which such bonding cannot occur, is as active and selective as HBB. It should be pointed out that structural modification of HBB has yielded some derivatives which are even more active, but not more selective, than HBB (*14*, *62*).

One of the outstanding achievements of modern virology is the grouping of hundreds of different animal viruses into about eight major groups, on the basis of their fundamental physical and chemical properties (*2*, *63*). The fact that HBB, and also guanidine, inhibit replication only of picornaviruses (*18–23*, *27*, *51*) indicates that these compounds "recognize" some specific physical-chemical feature of the susceptible viruses. They do not act by inhibiting some vital metabolic activity of host cells, because if they did, the multiplication of several groups of viruses would be affected by these compounds. Among the HBB-insusceptible viruses listed in Table 1 (*18*) are some which contain DNA (poxvirus, herpes simplex virus, the adenoviruses) and some which contain RNA (myxoviruses, arthropod-borne viruses, reoviruses, and a number of picornaviruses). Thus, sensitivity to HBB or guanidine is a property of certain, but by no means all, RNA viruses.

Although the actions of HBB and guanidine are in many ways similar, there is evidence that their sites of action are not identical. As may be seen in Table 2 (*20*), the spectra of virus-inhibiting action of HBB and guanidine overlap to a considerable extent but are not identical. There are a number of picornaviruses that are sensitive to guanidine but insusceptible to HBB. Many Coxsackie A viruses are sensitive to the former and insusceptible to the latter, as are some rhinoviruses. Thus, the spectrum of virus-inhibiting action of guanidine is broader than that of HBB. Some

Table 1. Spectrum of virus-inhibiting activity of HBB.

| Sensitive | Resistant |
|---|---|
| Picornaviruses (mostly enteroviruses) | Poxvirus |
| | Herpes simplex virus |
| | Adenoviruses |
| | Myxoviruses |
| | Arthropod-borne viruses |
| | Reoviruses |
| | Picornaviruses (mostly rhinoviruses; also some enteroviruses and others) |

Table 2. Inhibition of replication of picornaviruses by guanidine and HBB. Polio, Coxsackie A and B, and ECHO viruses make up the enterovirus group of picornaviruses; rhinoviruses constitute a second group. Mengovirus has not yet been placed into a group.

| Guanidine-sensitive | | Guanidine-resistant | |
|---|---|---|---|
| HBB-sensitive | HBB-resistant | HBB-sensitive | HBB-resistant |
| Polioviruses | Coxsackie A | | Rhinoviruses |
| Coxsackie A | Rhinoviruses | | ECHO 22, 23 |
| Coxsackie B | | | Mengovirus |
| ECHO | | | |

Table 3. Cellular activities not affected, or only slightly affected, by HBB.

| |
|---|
| *Biosynthetic process* |
| Incorporation of adenosine-8-$C^{14}$ or uridine-$H^3$ into RNA |
| Incorporation of $C^{14}$-L-alanine or $C^{14}$-L-leucine into proteins |
| *Energy metabolism* |
| Oxygen consumption |
| Glucose utilization |
| Lactic acid production |
| *Cell growth* |
| Rate of cell division |

picornaviruses are insusceptible to both HBB and guanidine. The fact that HBB and guanidine can "recognize" differences in the genetic and chemical fine structure among picornaviruses makes it possible to subdivide the picornaviruses into the three groups shown in Table 2. This grouping has many interesting aspects from the standpoint of biology, since the groups correspond (*14, 18, 20*) in a number of ways to the previously recognized groupings among the small lipid-free RNA viruses.

Guanidine and HBB are virus-specific in their action also in the sense that they do not inhibit cellular metabolic activities or growth at concentrations which are markedly inhibitory for the reproduction of sensitive viruses (*18, 24, 28, 51*). There is much direct evidence, summarized in Table 3, that HBB has no significant effects on cellular biosynthetic or energy-yielding processes. There is similar, though less extensive, evidence with respect to guanidine.

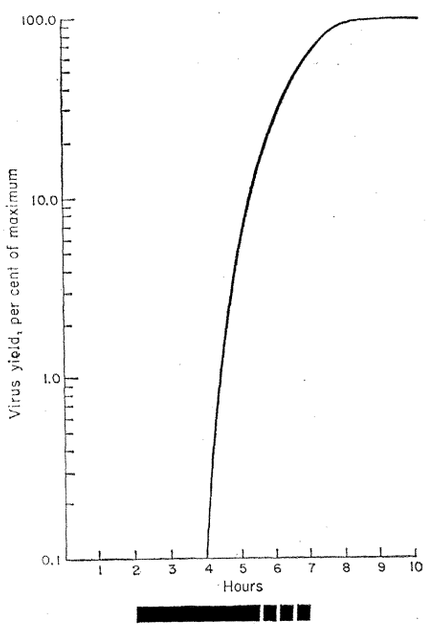



Fig. 3. Time course of the HBB-sensitive process in reproduction of ECHO 12 virus. (Solid bar) established duration; (solid bar plus broken bar) probable duration. [After Eggers and Tamm (*24*)]

As indicated in Table 4, HBB and guanidine prevent virus-directed synthesis of protein and RNA (*6, 24–28*). Neither compound inhibits adsorption of virus to the cell surface (*18, 24, 27*), and there is no evidence that either affects the release of virus.

In virus replication, the HBB-sensitive process begins after the initial steps in virus-cell interaction have been completed (*24*). The time course of the phase of sensitivity to HBB in the replication cycle of the enterovirus ECHO 12 is shown in Fig. 3 (*24*). The point at which virus replication becomes inhibitable by the compound is the mid-point of the latent period; that is, the presence of the compound during the first half of the latent period has no effect on the replication cycle, but when the compound is present at any time thereafter, virus replication does not proceed. Indeed, the process of virus replication remains inhibitable by HBB throughout the phase of exponential increase in virus.

The time course of the HBB-sensitive process in the replication of another enterovirus, Coxsackie A9, has been found to be similar to that for ECHO 12 (*26*). Somewhat different results have been obtained with guanidine. Crowther and Melnick reported (*27*) that the replication of poliovirus 1 becomes inhibitable by guanidine very shortly after infection of the cells. This is also true of Coxsackie A9 (*64*). With both viruses, the process of viral replication remains inhibitable by guanidine throughout the phase of exponential increase.

Of central importance for an understanding of the mechanism of action of HBB and guanidine is the fact that both compounds prevent the appearance in poliovirus-infected cells of the virus-induced RNA polymerase (*6*). The compounds have no effect on the activity of the enzyme system in vitro. If, as the evidence suggests, the virus-induced RNA polymerase is in fact the enzyme system responsible for synthesis of virus RNA, then it is clear that synthesis of virus RNA cannot proceed in a cell in which production of the necessary enzyme system is inhibited. One would also expect to find that the viral coat protein would not be made. The experiments which have been carried out show just this. We have found that HBB prevents both the synthesis of infective viral RNA and the incorporation of tritiated uridine into virus-directed RNA (*24, 25*). We have found, also, that HBB inhibits the synthesis of viral coat protein, as measured by complement fixation (*24*). In experiments in which different techniques were used, guanidine showed similar effects (*26–28*).

Table 4. Effects of HBB on steps in enterovirus replication.

| Step | Effect |
|---|---|
| Adsorption of virus to cells | None |
| Penetration and eclipse | None |
| Appearance of virus RNA polymerase | Inhibited |
| Synthesis of virus RNA | Inhibited |
| Synthesis of virus protein | Inhibited |
| Assembly of virus particles | No direct effects known |
| Release | No direct effects known |

Guanidine and HBB not only inhibit the production of virus-induced RNA polymerase and of virus constituents in infected cells but also markedly lessen virus-induced cell damage (see Fig. 4) (*18, 21, 27, 51, 53*). That guanidine partially prevents virus-induced depression of cellular RNA synthesis may or may not be significant in this connection (*28, 65*). The fact that guanidine does not prevent (*65, 66*) the rapid virus-induced depression in the synthesis of cellular protein (*45*) suggests that such depression is not a direct cause of the cytopathic effects of the virus, since these effects can be largely prevented by guanidine.

An important aspect of the inhibitory effect of HBB is its complete reversibility (*25, 53*); virus replication may be kept in abeyance for many hours by treatment of infected cells with HBB, but if the compound is removed, virus replication proceeds in the usual manner, as if the compound had never been present (*24*).

## Drug Resistance and Drug Dependence

When cultures infected with virus sensitive to HBB or guanidine are incubated in the presence of either of these compounds for a long time, virus "breakthrough" ultimately occurs: virus multiplies, and the particles produced are no longer highly sensitive to the drug but are in fact resistant (*18, 67*). The speed with which drug resistance develops depends on the virus and the compound used, and also on the con-

centration of each in the inoculum. "Breakthrough," with emergence of resistant mutants, occurs sooner with relatively large inocula of virus and low concentrations of inhibitor. In general, breakthrough with polioviruses occurs sooner in the presence of HBB than in the presence of guanidine, whereas with Coxsackie B and ECHO viruses the relationship is reversed: HBB causes more prolonged suppression of these viruses than does guanidine. When both compounds are present, the virus-suppressing effect of the combination is greater than the effect of either compound at double concentration (*20*). This may be explained at least in part by the finding that only partial cross resistance has been found between HBB and guanidine (*20*). Recent experiments, however, have also shown evidence of synergism in the virus-inhibiting actions of HBB and guanidine (*64*).

The HBB-resistant enterovirus mutants are stable in that the level of drug resistance in clonal populations derived from a single resistant virus particle does not change with repeated passage (*14*, *18*). Clonal populations which show different levels of resistance have been isolated.

Figure 5 illustrates the emergence of resistant Coxsackie A9 virus in the presence of HBB at a concentration of 22 micrograms per milliliter (*18*). At a concentration of 49 micrograms per milliliter, HBB completely suppressed the Coxsackie A9 strain used. Critical experiments have not yet been made to determine whether the resistance is drug-induced or whether the resistant mutants appear spontaneously. However, there is reason to think that HBB and guanidine may indeed induce or facilitate mutation to drug resistance. As may be seen in Fig. 5, a resistant population emerged in the presence of HBB when fewer than 100 infective units of sensitive wild-type parent virus were inoculated. The emergence of a resistant population has been regularly observed in many such experiments. Plating experiments with the parent virus population have shown that, if the parent population contains any resistant particles at all, they are present in a proportion smaller than 1 in 10,000. Thus, it is probable that HBB not only acted as a selective agent in the experiment shown in Fig. 5 but that it, in fact, caused the initial appearance of the drug-resistant mutant particles. In contrast to many mutagens, such as x-ray or methylating agents, HBB, even at high concentration, has no direct in-

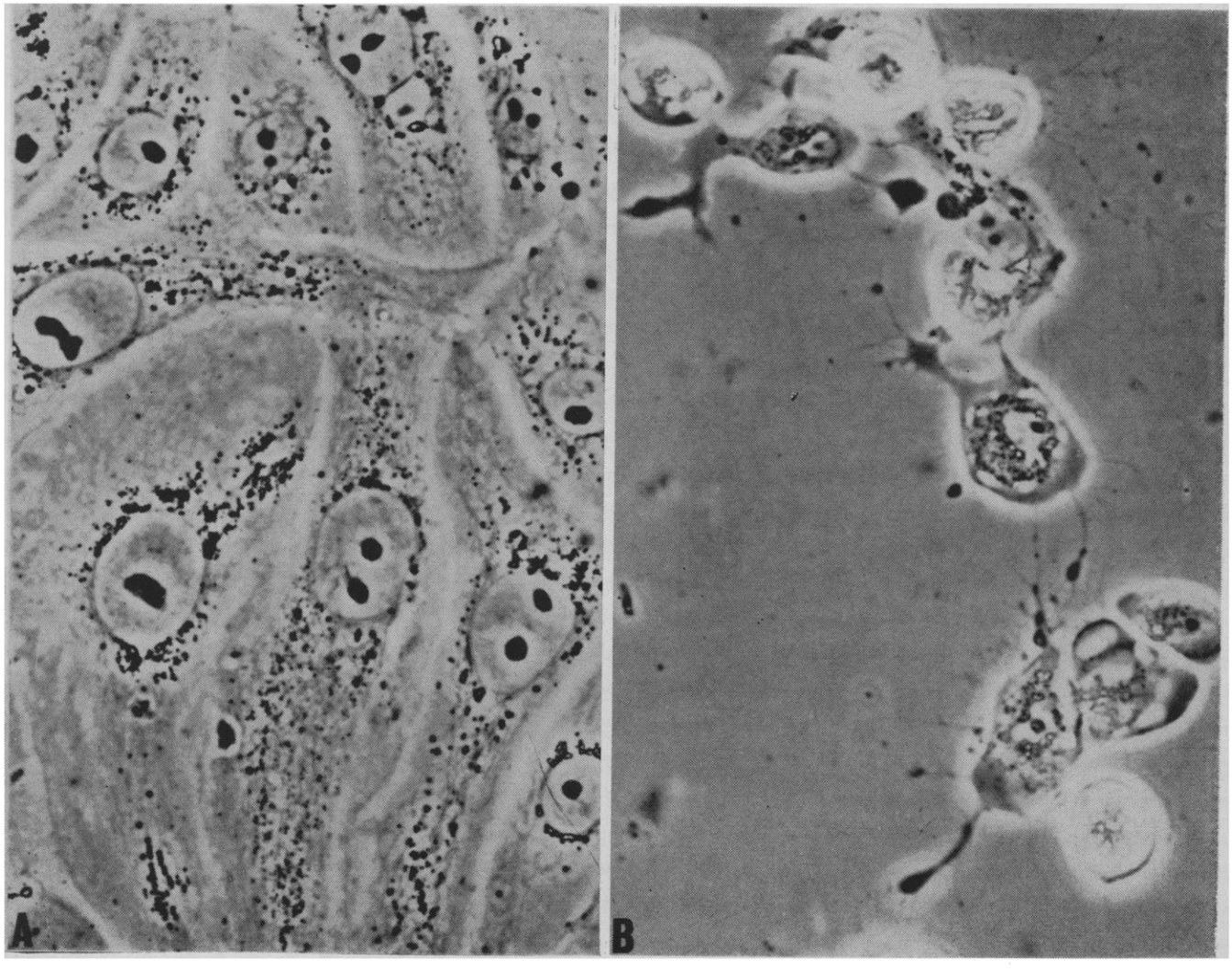



Fig. 4. Effect of HBB on virus-induced cell damage. Monolayer cultures of monkey kidney cells were infected with Coxsackie B4 virus (ten infective units per cell) and incubated at 37°C. (*A*) Culture treated with HBB (49$\mu$g/ml); the cells appear essentially normal. (*B*) Culture not treated with HBB; the cells are markedly damaged. (Phase contrast photomicrograph of unfixed-unstained cells, taken at 48 hours; magnification, $\sim \times 715$.) [Rostom Bablanian, Rockefeller Institute]

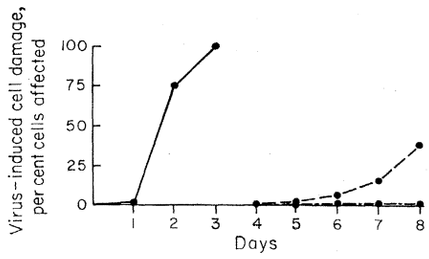



Fig. 5. Emergence of resistant Coxsackie A9 virus on prolonged incubation of infected cultures in the presence of HBB. (Solid line) No compound present; (dashed line) HBB present (22 $\mu$g/ml); (dashed and dotted line) HBB present (49 $\mu$g/ml). [Eggers and Tamm (*18*)]

activating effect on virus particles (*18*, *53*) or on infective viral RNA (*24*). If HBB does have direct effects on viral RNA which lead to mutation, they must be specific and restricted.

Of great interest is the fact that, in addition to resistant mutants, drug-dependent mutants of enteroviruses have been isolated (*68–71*). Thus viruses, like bacteria, can exist in the three states of drug sensitivity, drug resistance, and drug dependence. Studies with the HBB-dependent mutant of Coxsackie A9 virus have shown that infective RNA extracted from the HBB-dependent virus is also HBB-dependent (*69*, *70*). Clearly, HBB dependence is a property of the genetic material of the viruses; the RNA of dependent virus requires the compound for the expression of its genetic and reproductive potentialities. Similar results have been obtained with the guanidine-dependent mutant of poliovirus 1 (*72*).

Single-cycle growth characteristics of the HBB-dependent Coxsackie A9 variant, growing in the presence of an optimum concentration of HBB, are on the whole similar to those of the HBB-sensitive parent population growing in the absence of the compound (*70*). The latent period of the dependent virus is only slightly longer, the exponential increase somewhat slower, and the maximum yield of virus particles per cell is 10-percent lower than in the growth of the HBB-sensitive virus.

In a variety of experiments, sensitivity and dependence manifest themselves as the precise opposites of one another. As Fig. 6 shows, there is a striking similarity in the concentrations of HBB required for maximum growth of HBB-dependent virus or for marked inhibition of growth of the HBB-sensitive parent virus (*69*, *70*). In addition, the time course of the drug-dependent process in the replication of the dependent mutant is closely similar to the time course of the drug-sensitive process in the replication of the sensitive parent virus (*26*). The compound is not required during the first half of the latent period, but after that, replication proceeds only if the compound is present. Indeed, the compound is required also during the phase of exponential increase in virus. As for the biochemical mechanism of dependence, it appears that dependence is indeed a counterpart phenomenon of sensitivity, in that, whereas HBB prevents synthesis of viral RNA of drug-sensitive virus, it is required for replication of the RNA of drug-dependent mutants (*26*). Similar results were obtained recently with guanidine-dependent poliovirus in HeLa cells (*26*), and, in addition, it was shown in this system that drug-dependent virus requires the compound for production of a virus-induced RNA polymerase activity in the infected cells (*6*).

Experiments with drug-dependent or drug-resistant mutants of poliovirus were undertaken in order to secure additional evidence as to the virus-specific nature of the RNA polymerase which appears in the cytoplasm of poliovirus-infected cells (*6*). The poliovirus–HeLa cell system was used for reasons of convenience. The enzyme experiments require fairly large amounts of starting material—that is, infected cells—which can be readily procured by using a line of HeLa cells adapted to growth in suspension. The guanidine-resistant mutant of poliovirus 1 that was used multiplied to high yields in the presence of 100 micrograms per milliliter of guanidine; the dependent mutant required guanidine for replication. As is shown in Table 5, virus-induced RNA polymerase was not demonstrable in guanidine-treated cells which had been infected with poliovirus 1 of the drug-sensitive wild type (*6*). The virus-induced polymerase activity was demonstrable in cells infected with the drug-resistant virus, regardless of whether guanidine was present in the growth medium. Finally, the drug-dependent virus required guanidine for enzyme production. The enzyme did not require guanidine for activity in the cell-free system in which it was assayed.

Certain genetic aspects of HBB dependence are of special interest. The rate of back mutation to HBB independence is remarkably high (*69*, *70*). When HBB-dependent virus is multiplying in the presence of the compound, one out of every 100 to 600 particles produced is HBB-independent. For the HBB-dependent ECHO 13 virus, the figure is lower: one out of every 1000 to 5000 particles produced is drug-independent. Back mutation is toward either HBB resistance or HBB sensitivity. The relative frequency of occurrence of each is being investigated through the use of unsubstituted benzimidazole, a compound which, at a concentration of 120 micrograms per milliliter, fully supports the growth of dependent virus but does not inhibit that of sensitive virus. This characteristic has made it possible to grow HBB-dependent virus in an environment which allows multiplication of any HBB-sensitive virus that arises through back mutation. In short, these conditions do not selectively favor the growth of resistant back mutants.

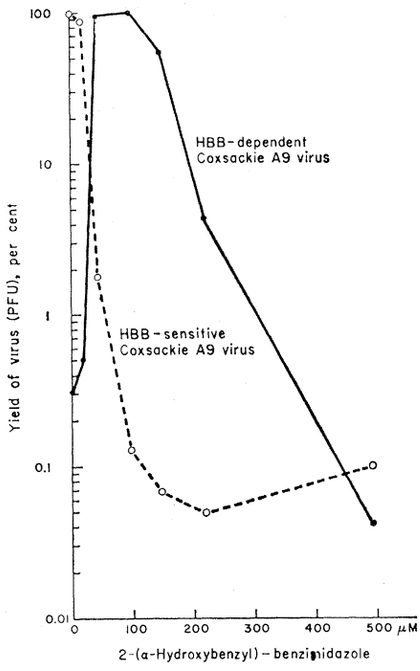



Fig. 6. Effect of the concentration of HBB on the growth of HBB-dependent and HBB-sensitive Coxsackie A9 virus. [Eggers and Tamm (*70*)]

In addition to unsubstituted benzimidazole, 5-methyl-2-D-ribobenzimidazole (*60*) also supports full growth of the HBB-dependent mutant (*69*, *70*). Since neither of these compounds specifically inhibits the replication of drug-sensitive enteroviruses, we conclude that the structural requirements for inhibiting multiplication of wild-type viruses or for enhancing multiplication of mutant strains are not identical.

## Inhibition of Poxviruses by Thiosemicarbazones

Although it has been known for over 10 years that certain thiosemicarbazones can protect mice against death from experimentally induced infection with vaccinia virus (*73*), it is only recently that some understanding has been gained of the mechanism whereby some of them act (*29, 30, 74*). Moreover, recent work with the *N*-ethyl and *N*-methyl derivatives of isatin β-thiosemicarbazone suggests that these derivatives might prove useful in the chemoprophylaxis and possibly in the therapy of poxvirus infections (*75*).

Thompson and his co-workers and Bauer and his co-workers demonstrated that isatin β-thiosemicarbazone (Fig. 7) is inactive against entero-, myxo-, arthropod-borne, herpes, and rabies viruses (*76*); it is nontoxic at concentrations which inhibit the replication of poxviruses (*74*). It inhibits the maturation of vaccinia virus, a poxvirus, but has no apparent effect on the synthesis of its DNA and protein (*29, 30*). However, studies on the time course of the phase of drug sensitivity have shown that the process of virus replication becomes inhibitable by isatin β-thiosemicarbazone shortly after infection of cells by the virus—that is, long before the assembly of virus particles begins (*29, 30*). The drug-sensitive process is not restricted to the early part of the multiplication cycle: it continues for many hours. Virus particles produced in the presence of isatin β-thiosemicarbazone are noninfective, and morphologically they resemble immature forms of vaccinia virus; they are nearly spherical, and though they contain material in their interiors, they do not have a dense nucleoid structure. Isatin β-thiosemicarbazone thus specifically interferes with a step in poxvirus replication which is required for maturation of the virus.

## Inhibition of DNA Viruses by Halogenated Deoxyuridines

For many years there was no evidence to support the contention that structural analogs of naturally occurring metabolites might be discovered which would possess selective virus-inhibiting activity. Recently, however, it was shown that 5-iodo-2′-deoxyuridine (Fig. 8) (*77*) is effective in treating eye infections induced experimentally with two DNA-containing viruses, herpes simplex and vaccinia (*32*). This compound has indeed proved useful in the treatment of herpes keratitis in man (*78*).

Table 5. Effect of guanidine on the appearance of the virus-induced RNA polymerase in cells infected with drug-resistant or drug-dependent virus mutants.

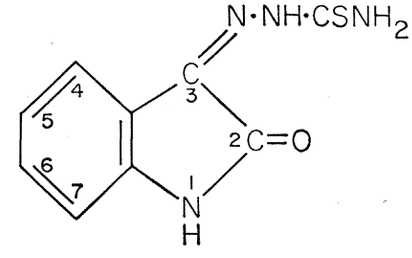
| Poliovirus type 1 | Guanidine (100 μg/ml) present during infection | Appearance of virus RNA polymerase |
|---|---|---|
| Sensitive (wild type) | No | Yes |
| Sensitive (wild type) | Yes | No |
| Resistant mutant | No | Yes |
| Resistant mutant | Yes | Yes |
| Dependent mutant | No | No |
| Dependent mutant | Yes | Yes |

In 1959 Alison Newton and one of us (I.T.) found (*31*) that the 5-fluoro-derivative of 2′-deoxyuridine (Fig. 8) was, on a weight basis, the most active inhibitor of virus multiplication so far discovered. At a concentration of $10^{-8}M$ or 0.0025 micrograms per milliliter, 5-fluoro-2′-deoxyuridine caused partial inhibition of reproduction of herpes simplex virus and prevented the increase in DNA which is regularly observed in HeLa cells after infection with herpes virus. At $10^{-7}M$, the virus-inhibiting effect of the compound was marked. At this concentration, the compound did not have detectable toxic effects on the appearance of cells. However, it did reduce cell division by 80 percent (*79*). Infected cells which produced little or no virus in the presence of the compound nevertheless showed the cytopathic effect characteristic of this virus (*31*). The fluoro compound is not therapeutically effective in herpes simplex infections of the eye, where the iodo- and bromo-derivatives are useful (*32*); this again shows in a striking way the importance of structural modification of promising compounds.

The biochemical basis for the selective action of the iodo- and bromo-derivatives on the reproduction of herpes and vaccinia viruses is not yet clear. The iodo- and bromo- derivatives inhibit the utilization of thymine compounds but not their syntheses, whereas the fluoro- compound inhibits the synthesis of thymidine monophosphate (*77*). The iodo- and bromo-derivatives are incorporated into DNA, but the fluoro- derivative appears not to be. The extent of the incorporation of the bromo- or iodo- compound into cellular DNA and of subsequent chromosome damage depends markedly on the concentration of the compound. At a concentration of $10^{-6}M$, the bromo-derivative does not affect the growth rate of cells during two to two and a half cell divisions but inhibits the production of herpes virus in cell culture by 97 percent (*33*). In the presence of 5-bromo-2′-deoxyuridine, cells infected with vaccinia virus yield large quantities of noninfectious, malformed virus particles; this may be a consequence of the incorporation of the compound into the virus DNA (*34*). It is also possible, however, that the iodo- and bromo-derivatives block viral DNA synthesis by acting selectively on a virus-specific enzyme system, such as a DNA polymerase, which may be involved in the synthesis of viral DNA. (*32*, and A. Newton, personal communication.)

## Conclusions

Guanidine and HBB specifically inhibit the reproduction of many small, lipid-free RNA viruses which belong to a single large group, the picornaviruses. The group includes polio-, Coxsackie, ECHO, rhino-, and a number of other viruses. Guanidine and HBB have no significant effects on the multiplication of viruses belonging to other groups, or on the normal metabolic activities of cells. These compounds block the synthesis of virus-induced RNA polymerase and of viral RNA and viral coat protein of drug-sensitive picornaviruses.




**Isatin β-thiosemicarbazone**

Fig. 7. Structure of a specific inhibitor of poxvirus reproduction.

A hypothesis to explain these findings may be formulated as follows. On infection of cells with a picornavirus, viral RNA, acting as messenger RNA, directs the production of an RNA polymerase which functions in the synthesis of new viral RNA. The new viral RNA provides the messenger RNA for the synthesis of viral coat protein. Guanidine and HBB block the synthesis of virus-induced RNA polymerase and thereby prevent the manufacture of new viral RNA and viral coat protein.

The fact that both poliovirus and Mengovirus can direct the synthesis of virus RNA polymerase but that only one of the two—poliovirus—is sensitive to HBB and guanidine raises an interesting question: Is the difference in sensitivity due to a critical difference in the biochemical mechanisms of synthesis of the two RNA polymerases, or is the mechanism of synthesis the same but the structure of the nucleic acid of the two viruses critically different? This question applies with even greater force to the drug-resistant mutants of originally drug-sensitive enteroviruses. There is reason to believe that picornaviruses largely utilize the cellular machinery for protein synthesis for the synthesis of virus-directed proteins. It seems unlikely that in the protein-synthesizing mechanism in the cells there exist alternative pathways for the drug-sensitive, drug-resistant, and drug-dependent variants of a single virus.

A more attractive idea is the suggestion that differences in the structure of the nucleic acid of picornaviruses are directly involved in the variable response of picornaviruses to HBB and guanidine. There is much evidence that the action of these compounds depends on the genetic, and therefore chemical, fine structure of the RNA in picornaviruses. Whether only the sequence of nucleotides is concerned, or whether some aspects of the secondary structure of viral RNA are also involved, is not clear, but this information is not essential to the general argument. One may visualize the RNA of picornaviruses as having three structural states, which affect its function. In one state it is sensitive to HBB or guanidine; in another, it is drug-resistant; and in the third, it depends on, or requires, the drug. It is not possible to decide, on the basis of the available information, whether the effects of the compounds are produced through direct combination with the viral nucleic acid itself or through combination with a component in infected cells—for example, the ribosome—with which the viral RNA reacts. If HBB and guanidine combine with viral nucleic acid, they may inhibit not only the messenger function of viral RNA but also its gene function. There can be little doubt that viral RNA must, in one way or another, serve as a template in its own replication. There is no direct evidence that HBB and guanidine inhibit such a template function. Theoretically, this is an interesting possibility. Selective control of the intimate process of gene function has been discussed previously by Luria (*80*).

An alternative hypothesis has recently been proposed (*81*) to explain the specific effects of guanidine on the production of virus-induced RNA polymerase in active form. Lwoff and his co-workers (*81*) have suggested that guanidine may inhibit the formation of the active polymerase from a precursor protein. The drug-dependent virus may require the compound for successful conversion of precursor protein into active enzyme. This interesting idea is compatible with data which are at present available on the effects of guanidine on the production of virus-induced RNA polymerase.

These various hypotheses can be tested experimentally.

One should note that, although the actions of HBB and guanidine are in many ways similar, they are by no means identical. Some Coxsackie A and rhino- viruses are sensitive to guanidine but completely insensitive to HBB. Polioviruses are on the whole more sensitive to guanidine than to HBB, whereas Coxsackie B and ECHO viruses are more sensitive to HBB than to guanidine. Guanidine inhibits some as yet unidentified process which takes place during the first half of the latent period of the viral growth cycle; HBB has no effect during this period. Once the primary sites of action of HBB and guanidine have been established with certainty, all these findings should be readily interpretable.

The study of virus-specific processes in the reproduction of animal viruses has barely begun, but already new information has been gained. The occurrence in picornavirus-infected cells of

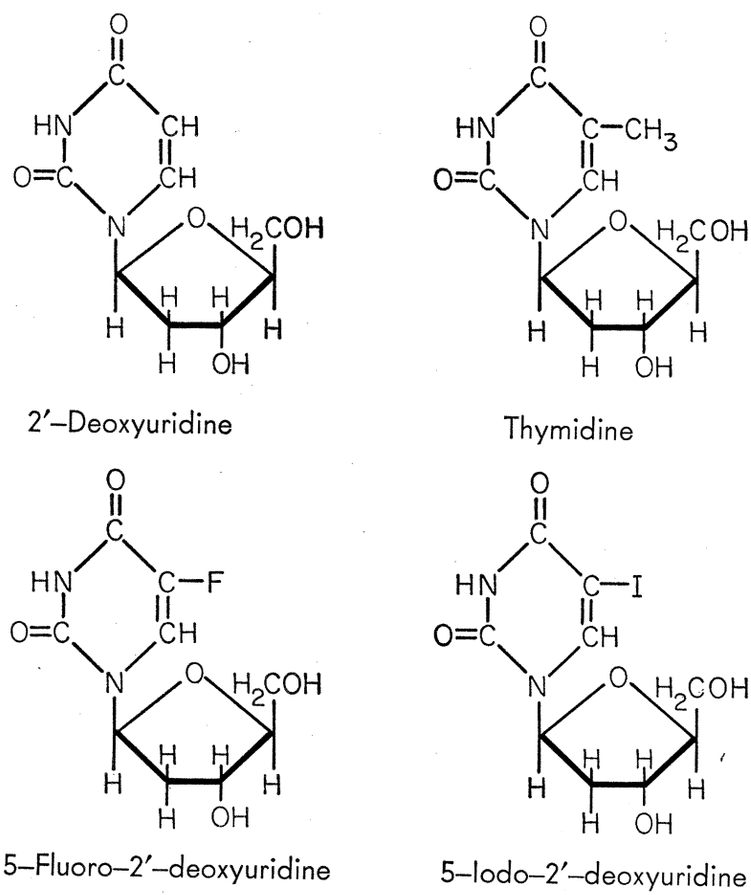



Fig. 8. Structures of two metabolic precursors in DNA synthesis, and of two structural analogs.

a virus-induced enzyme, which has the characteristics of an RNA-dependent polymerase, has been described. Another finding which concerns viral RNA, but RNA of a very different virus, is the observation that in reovirus the genetic material is an uncommonly large, double-stranded RNA (*82*). The process of its synthesis probably also has special features. Lest it be thought that these findings apply only to animal viruses, it should be pointed out that the RNA-containing bacteriophage also directs the synthesis of a new RNA polymerase (*83*), and the wound-tumor virus of plants also has a double-stranded RNA (*82*). These are examples of remarkable similarity in the molecular biology of viruses affecting widely different hosts.

To summarize, virus-specific processes occur in the reproduction of animal viruses, and chemical inhibitors are useful in their study. Specific chemical inhibition of the intimate mechanism of synthesis of virus nucleic acid offers means of controlling virus diseases (*16*, *84*).

### References and Notes


1. S. S. Cohen, *Federation Proc.* **20**, 641 (1961); A. Kornberg, *Enzymatic Synthesis of DNA* (Wiley, New York, 1961).
2. A. Lwoff, R. Horne, P. Tournier, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 51 (1962).
3. S. Brenner, F. Jacob, M. Meselson, *Nature* **190**, 576 (1961); T. Kano-Sueoka and S. Spiegelman, *Proc. Natl. Acad. Sci. U.S.* **48**, 1942 (1962).
4. D. Nathans, G. Notani, J. H. Schwartz, N. D. Zinder, *Proc. Natl. Acad. Sci. U.S.* **48**, 1424 (1962); J. Warner, M. J. Madden, J. E. Darnell, *Virology* **19**, 393 (1963).
5. D. Baltimore and R. M. Franklin, *Biochem. Biophys. Res. Commun.* **9**, 388 (1962).
6. D. Baltimore, H. J. Eggers, R. M. Franklin, I. Tamm, *Proc. Natl. Acad. Sci. U.S.* **49**, 843 (1963).
7. S. Kit, D. R. Dubbs, L. J. Piekarski, *Federation Proc.* **22**, 645 (1963).
8. I. Tamm, K. Folkers, F. L. Horsfall, Jr., *Yale J. Biol. Med.* **24**, 559 (1952).
9. I. Tamm, *ibid.* **29**, 33 (1956).
10. D. W. Woolley, *A Study of Antimetabolites* (Wiley, New York, 1952).
11. R. E. F. Matthews and J. D. Smith, *Advan. Virus Res.* **3 (1955)**, 49 (1955).
12. I. Tamm, *Symp. Soc. Gen. Microbiol.* **8 (1958)**, 178 (1958).
13. R. Markham, *ibid.*, p. 163.
14. I. Tamm and H. J. Eggers, in *International Symposium of Chemotherapy, 2nd, Naples* (1961), (Karger, New York, 1963), pt. 2, p. 88.
15. E. W. Hurst and R. Hull, *Pharmacol. Rev.* **8**, 199 (1956); M. Staehelin, *Progr. Med. Virol.* **2 (1959)**, 1 (1959); W. Cutting and A. Furst *Antibiot. Chemotherapy* **8**, 441 (1958).
16. I. Tamm, *Antibiot. Chemotherapy* **12**, 437 (1962).
17. J. L. Melnick *et al.*, *Virology* **19**, 114 (1963).
18. H. J. Eggers and I. Tamm, *J. Exptl. Med.* **113**, 657 (1961).
19. ———, *Virology* **13**, 545 (1961).
20. I. Tamm and H. J. Eggers, *ibid.* **18**, 439 (1962).
21. W. A. Rightsel *et al.*, *Science* **134**, 558 (1961).
22. B. Loddo, *Boll. Soc. Ital. Biol. Sper.* **38**, 8 (1962).
23. S. Toyoshima, T. Ueda, T. Tsuji, Y. Seto, J. Nomoto, *Chem. Pharm. Bull. Tokyo* **11**, 5 (1963).
24. H. J. Eggers and I. Tamm, *Virology* **18**, 426 (1962).
25. ———, *Nature* **197**, 1327 (1963).
26. H. J. Eggers, E. Reich, I. Tamm, *Proc. Natl. Acad. Sci. U.S.* **50**, 183 (1963).
27. D. Crowther and J. L. Melnick, *Virology* **15**, 65 (1961).
28. J. J. Holland, *Proc. Natl. Acad. Sci. U.S.* **49**, 23 (1963).
29. K. B. Easterbrook, *Virology* **17**, 245 (1962).
30. M. K. Bach and W. E. Magee, *Proc. Soc. Exptl. Biol. Med.* **110**, 565 (1962).
31. A. A. Newton and I. Tamm, in preparation.
32. H. E. Kaufman, in *Perspectives in Virology*, M. Pollard, Ed. (Harper, New York, 1960), vol. 3, p. 90; ———, *Proc. Soc. Exptl. Biol. Med.* **109**, 251 (1962); ———, A. B. Nesburn, E. D. Maloney, *Virology* **18**, 567 (1962).
33. P. Siminoff, *Intern. Congr. Microbiol., 8th, Montreal* **1962**, 81 (1962).
34. K. B. Easterbrook and C. I. Davern, *Virology* **19**, 509 (1963).
35. F. L. Schaffer and C. E. Schwerdt, *Advan. Virus Res.* **6 (1959)**, 159 (1959).
36. A. Lwoff, *Biological Order* (Massachusetts Institute of Technology Press, Cambridge, 1962).
37. A. Klug and D. L. D. Caspar, *Advan. Virus Res.* **7**, 225 (1960).
38. J. V. Maizel, Jr., *Federation Proc.* **22**, 645 (1963); personal communication.
39. R. M. Franklin and D. Baltimore, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 175 (1962).
40. ———, *Symp. Fundamental Cancer Res. 17th, Houston, 1963* (in press).
41. J. J. Holland and B. H. Hoyer, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 101 (1962).
42. P. W. Choppin and L. Philipson, *J. Exptl. Med.* **113**, 713 (1961); L. Philipson and P. W. Choppin, *Virology* **16**, 405 (1962); L. Philipson and S. Bengtsson, *ibid.* **18**, 457 (1962).
43. S. Dales, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 132 (1962).
44. D. Baltimore and R. M. Franklin, *Proc. Natl. Acad. Sci. U.S.* **48**, 1383 (1962); J. J. Holland, *Biochem. Biophys. Res. Commun.* **9**, 556 (1962).
45. E. F. Zimmerman, M. Heeter, J. E. Darnell, *Virology* **19**, 400 (1963).
46. J. E. Darnell, Jr., and H. Eagle, *Advan. Virus Res.* **7**, 1 (1960).
47. E. Reich, R. M. Franklin, A. J. Shatkin, E. L. Tatum, *Proc. Natl. Acad. Sci. U.S.* **48**, 1238 (1962); A. J. Shatkin, *Biochim. Biophys. Acta* **61**, 310 (1962).
48. J. M. Kirk, *Biochim. Biophys. Acta* **42**, 167 (1960); J. Kawamata and M. Imanishi, *Biken's J.* **4**, 13 (1961); H. M. Rauen, H. Kersten, W. Kersten, *Z. Physiol. Chem.* **321**, 139 (1960); L. D. Hamilton, W. Fuller, E. Reich, *Nature* **198**, 538 (1963).
49. M. N. Goldstein, I. J. Slotnick, L. J. Journey, *Ann. N.Y. Acad. Sci.* **89**, 474 (1960); A. J. Shatkin, E. Reich, R. M. Franklin., E. L. Tatum, *Biochim. Biophys. Acta* **55**, 277 (1962); T. Tamaoki and G. C. Mueller, *Biochem. Biophys. Res. Commun.* **9**, 451 (1962); I. Merits, *ibid.* **10**, 254 (1963); R. M. Franklin, *Biochim. Biophys. Acta* **72**, 555 (1963).
50. I. H. Goldberg and M. Rabinowitz, *Science* **136**, 315 (1962); ——— and E. Reich, *Proc. Natl. Acad. Sci. U.S.* **48**, 2094 (1962); E. Harbers and W. Müller, *Biochem. Biophys. Res. Commun.* **7**, 107 (1962).
51. B. Loddo, W. Ferrari, G. Brotzu, A. Spanedda, *Nature* **193**, 97 (1962).
52. A. C. Hollinshead and P. K. Smith, *J. Pharmacol. Exptl. Therap.* **123**, 54 (1958).
53. I. Tamm *et al.*, *J. Exptl. Med.* **113**, 625 (1961).
54. I. Tamm, *Bull. N.Y. Acad. Med.* **31**, 537 (1955); *J. Bacteriol.* **72**, 42 (1956).
55. D. A. J. Tyrrell and I. Tamm, *J. Immunol.* **75**, 43 (1955).
56. I. Tamm, M. M. Nemes, S. Osterhout, *J. Exptl. Med.* **111**, 339 (1960).
57. V. G. Allfrey, A. E. Mirsky, S. Osawa, *J. Gen. Physiol.* **40**, 451 (1957); V. G. Allfrey and A. E. Mirsky, *Proc. Natl. Acad. Sci. U.S.* **43**, 821 (1957).
58. I. Tamm, *Science* **120**, 847 (1954); ———, K. Folkers, C. H. Shunk, F. L. Horsfall, Jr., *J. Exptl. Med.* **99**, 227 (1954); I. Tamm, K. Folkers, C. H. Shunk, *J. Bacteriol.* **72**, 54 (1956).
59. N. Ikegami, S. Kato, J. Kamahora, *Biken's J.* **3**, 57 (1960).
60. I. Tamm, *Virology* **2**, 517 (1956); ——— and M. M. Nemes, *Federation Proc.* **16**, 435 (1957).
61. D. G. O'Sullivan and P. W. Sadler, *Nature* **192**, 341 (1961).
62. A. F. Wagner, P. E. Wittreich, A. Lusi, K. Folkers, *J. Org. Chem.* **27**, 3236 (1962).
63. C. H. Andrewes, *Advan. Virus Res.* **9**, 271 (1962).
64. H. J. Eggers and I. Tamm, *Nature* **199**, 513 (1963).
65. R. Bablanian, H. J. Eggers, I. Tamm, in preparation.
66. D. Baltimore, H. J. Eggers, I. Tamm, in preparation.
67. J. L. Melnick, D. Crowther, J. Barrera-Oro, *Science* **134**, 557 (1961).
68. H. J. Eggers, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 309 (1962).
69. ——— and I. Tamm, in *Abstr. Intern. Congr. Microbiol., 8th* (1962), p. 85.
70. ———, *Virology* **20**, 62 (1963).
71. B. Loddo, W. Ferrari, A. Spanedda, G. Brotzu, *Experientia* **18**, 518 (1962); N. Ledinko, *Cold Spring Harbor Symp. Quant. Biol.* **27**, 309 (1962); *Virology* **27**, 107 (1963).
72. B. Loddo, S. Muntoni, A. Spanedda, G. Brotzu, W. Ferrari, *Nature* **197**, 315 (1963).
73. D. Hamre, J. Bernstein, R. Donovick, *Proc. Soc. Exptl. Biol. Med.* **73**, 275 (1950); R. L. Thompson, M. L. Price, S. A. Minton, Jr., *ibid.* **78**, 11 (1951).
74. F. W. Sheffield, *Brit. J. Exptl. Pathol.* **43**, 59 (1962); B. P. Sagik and B. S. Wright, *Bacteriol. Proc.* **1962**, 140 (1962).
75. D. J. Bauer and P. W. Sadler, *Lancet* **1960-I**, 1110 (1960); W. Turner, D. J. Bauer, R. H. Nimmo-Smith, *Brit. Med. J.* **1962-I**, 1317 (1962).
76. R. L. Thompson, J. Davis, P. B. Russell, G. H. Hitchings, *Proc. Soc. Exptl. Biol. Med.* **84**, 496 (1953); R. L. Thompson, S. A. Minton, Jr., J. E. Officer, G. H. Hitchings, *J. Immunol.* **70**, 229 (1953); D. J. Bauer, *Brit. J. Exptl. Pathol.* **36**, 105 (1955); ——— and P. W. Sadler, *Brit. J. Pharmacol.* **15**, 101 (1960).
77. A. D. Welch, *Cancer Res.* **21**, 1475 (1961); E. C. Herrmann, Jr., *Proc. Soc. Exptl. Biol. Med.* **107**, 142 (1961).
78. H. E. Kaufman, E. L. Martola, C. Dohlman, *Arch. Ophthalmol.* **68**, 235 (1962).
79. E. F. Wheelock and I. Tamm, *J. Exptl. Med.* **114**, 617 (1961).
80. S. E. Luria, *Science* **136**, 685 (1962).
81. A. Lwoff and M. Lwoff, *Compt. Rend.* **256**, 5001 (1963); A. Lwoff, A. Koch, M. Lwoff, *Compt. Rend.*, in press.
82. P. J. Gomatos and I. Tamm, *Proc. Natl. Acad. Sci. U.S.* **49**, 707 (1963).
83. C. Weissmann, L. Simon, S. Ochoa, *ibid.*, p. 407; A. M. Kaye, P. J. Ortiz, J. T. August, *Federation Proc.* **22**, 463 (1963).


84. This study was aided by a grant from the National Foundation.