Chemotherapy of Virus Diseases Volume I

Section Editor

D. J. Bauer

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Section 61

CHEMOTHERAPY OF VIRUS DISEASES

Section Editor

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Beckenham

VOLUME I

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PREFACE

ANTIVIRAL chemotherapy began to develop much later than antibacterial chemotherapy, and has so far produced far fewer useful drugs, but the literature on the subject is already sufficiently extensive to necessitate the division of this work into two volumes in order to afford the coverage which the subject requires.

During recent years the term virus has become much more restricted in definition as knowledge has increased, and a number of agents at first considered to be viruses are now assigned to other groups. They are omitted from this work, and there will therefore be no account of the chemotherapy of the agents of the lymphogranuloma-psittacosis groups, rickettsias and other such organisms. From the historical point of view this was at one time a considerable loss, since these agents are sensitive to sulphonamides, tetracyclines and other drugs, and it was therefore felt that antiviral chemotherapy had already come into being. As a result of the reclassification of these organisms, it has been necessary to lay the foundations of the subject anew.

How this came about is described in the introduction. The first antiviral compound to be discovered had been synthesized years before, and it was necessary for a number of random events to take place before its potential was realized. Since then, antiviral chemotherapy has made further progress, but chance has always played a great part in the discovery of new classes of compound with antiviral activity.

In order to detect and quantitate antiviral activity, tests and assays are required, and although it has been necessary to devise these in accordance with the special features of virological methods, they still depend upon the fundamental principles which have been long established for the action of drugs in general.

Antiviral chemotherapy became of clinical significance in 1953, when methisazone and idoxuridine were first used in man at about the same time. At the time of writing, these two drugs and amantadine hydrochloride are the only antiviral agents which have a place in clinical medicine. It seems likely, however, that a period of expansion is at hand, which comes at a

time when antibacterial chemotherapy, although greatly in advance, seems to be dependent more on its past successes than its prospects of future achievements.

As there are so few antiviral drugs in use at present, it follows that there are not many virus diseases which are susceptible to specific chemotherapy or chemoprophylaxis. This book is therefore subdivided into chapters according to classes of compounds rather than types of disease. The authors have been closely associated with the discovery and development of the agents which they describe. Little editorial constraint has been imposed, and the authors have been encouraged to present their subject matter in the way which seems best to them, although this has necessarily led to some lack of uniformity in the layout of the individual chapters. The treatment is detailed and monographic in character, and an attempt has been made to review all significant publications in the individual fields. Clinical aspects have been emphasized as far as possible.

The work which led to the development of methisazone and idoxuridine is described in the chapters on Thiosemicarbazones and Purines and Pyrimidines. Amantadine hydrochloride and related compounds will be included in Volume 2. Separate chapters are devoted to HBB and guanidine, although they are not used clinically, since work on these compounds and derivatives occupies a very important place in the history of the development of antiviral chemotherapy and has been of great value in studying the molecular biology of the multiplication of the enteroviruses.

Interferon is not a chemotherapeutic agent in the strictest sense, but a chapter on virus-induced interferons has been included, since interferon affords an alternative approach to the control of virus infections which is still awaiting its practical fulfilment. Other types of interferon inducer, which are mainly chemical substances, will be dealt with in Volume 2.

In addition to the usual subject and author indexes the work is provided with a compound index, in which derivatives are listed alphabetically under the names of their parent compounds.

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INTRODUCTION TO ANTIVIRAL CHEMOTHERAPY

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HISTORY OF THE DEVELOPMENT OF THE ANTIVIRAL AGENTS

At the time of writing, the foundations of antiviral chemotherapy have been laid, and rapid progress is being made in opening up a field which has hitherto been more noteworthy for its disappointments than for its achievements. It has been marked also by extreme slowness of development. Beginning with the discovery of the sulphonamides in the 1930s, effective antibacterial agents were discovered in rapid succession and brought into clinical use; none of these agents were effective against true viruses, an unfortunate fact which seems to have engendered an atmosphere of pessimism in which the discovery of the first true antiviral agent, by Hamre, Bernstein and Donovick in 1950, virtually escaped attention. The field of antiviral chemotherapy also suffered severely at the outset at the hands of the taxonomist. The agents of the lymphogranuloma–psittacosis group were at first considered to be viruses, and the discovery of their susceptibility to sulphonamides gave good grounds for hope that an entry had been made into the antiviral field. This hope was speedily dashed by a reclassification of these agents which related them to the rickettsias, and they are not considered in the present work, which will be limited to a presentation of the chemotherapy of infections of man and animals caused by true viruses.

The first true antiviral agents which were discovered were the thiosemicarbazones. They were introduced into medicine by Domagk *et al.* (1946). These workers prepared the thiosemicarbazones of benzaldehyde and a number of its derivatives, and found that they were active against tubercle bacilli *in vitro* and in animals. Thiosemicarbazones are still used

in the treatment of tuberculosis at the present time, although their use has been restricted by their toxicity and by the subsequent discovery of other effective agents.

The discovery of an agent effective in one branch of microbiological science is usually followed by its trial in other branches, and Hamre, Bernstein and Donovick (1950) found that *p*-aminobenzaldehyde thiosemicarbazone was active against vaccinia infection in fertile eggs and mice, and the first example of a structure–activity analysis of antiviral activity in a quantitative examination of the effect of introducing various substituents into the benzene ring was provided subsequently by Hamre, Brownlee and Donovick (1951). Thompson, Price and Minton (1951) found that benzaldehyde thiosemicarbazone protected mice infected with vaccinia virus intracerebrally. Thus, *p*-aminobenzaldehyde thiosemicarbazone was the first antiviral agent to be discovered, and benzaldehyde thiosemicarbazone was the first antiviral agent to be found active in animals.

The study of the thiosemicarbazones was carried further by Thompson *et al.* (1953), who observed that the thiosemicarbazones of a number of cyclic aldehydes were highly effective in mice infected with vaccinia virus. This work was continued by Bauer (1955) and Bauer and Sadler (1960), and culminated in the demonstration by Bauer *et al.* (1963) of the prophylactic effect of 1-methylisatin 3-thiosemicarbazone in persons exposed to smallpox infection. This was the first time that a drug had been shown to be effective in the prevention of a virus disease in man. The same drug was also shown to be effective in the treatment of certain infective complications of smallpox vaccination (Bauer, 1965). Its antiviral spectrum was greatly extended by the discovery that it was also active against adenoviruses (Bauer and Apostolov, 1966).

The elucidation of the structure of vitamin B and the demonstration that it contained a benzimidazole residue led to the trial of benzimidazoles as antiviral agents, and to the discovery of 2,5-dimethylbenzimidazole by Tamm, Folkers and Horsfall (1952), a compound which inhibits the multiplication of types A and B influenza virus in the chorioallantoic membrane. In an extension of this work, derivatives of benzimidazole with a sugar moiety, which thus resembled vitamin B_{12} more closely, were also found to be effective against influenza virus. Compounds of this type were shown much later (Bucknall, 1967) to owe their antiviral action to an unspecific inhibitory effect, and no evidence has been presented that vitamin B_{12} is involved in virus metabolism. Nevertheless, the further investigation of this apparently false trail led to the discovery of

2-(α-hydroxybenzyl)benzimidazole, a compound which is very effective in inhibiting the multiplication of poliomyelitis virus and other enteroviruses in tissue culture. There have been no reports of any trials of compounds of this type in man. This must be ascribed to the fact that the concomitant development of effective poliomyelitis vaccines has virtually eradicated the disease and thus removed the opportunity for testing chemotherapeutic agents in its control and treatment, but this circumstance in no way detracts from the importance of the discovery of the benzimidazoles as a milestone in the history of antiviral activity.

A new direction in antiviral chemotherapy developed from the observation that 5-iodouracil had antiviral activity. This led Prusoff (1959) to synthesize the corresponding deoxyriboside, 5-iodo-2'-deoxyuridine (idoxuridine). This also had antibacterial activity, and a study of its mode of action indicated that it prevented the incorporation of thymidine into deoxyribonucleic acid. This postulated activity against DNA led to the supposition that it might have activity against DNA-containing viruses, and Herrmann (1961), using his recently developed plaque-inhibition test, showed that the compound was active against vaccinia and herpes viruses. Kaufman (1962) found that experimental herpetic keratitis in rabbits responded to the repeated instillation of a solution of idoxuridine, and was then able to show (Kaufman, Martola and Dohlman, 1962) that the compound was effective in the treatment of herpetic keratitis in man. This observation stimulated a spate of further work; much of this was insufficiently controlled, and not all of it was favourable, but it has now been firmly established that herpetic keratitis can be successfully treated with idoxuridine. At present, the drug is restricted to situations in which it can be used topically, as it is rapidly metabolized to inactive products after administration by the usual routes. Some success has been reported in the treatment of cutaneous herpes by local application of the drug dissolved in dimethylsulphoxide, and some effect has been claimed in the treatment of herpetic encephalitis by intravenous and intraarterial infusion. Idoxuridine is at present the standard treatment for herpetic keratitis, and its introduction into clinical practice is a very important landmark in antiviral chemotherapy.

An entirely different approach to the problem of finding effective antiviral agents has developed from studies of the phenomenon of virus interference. This has been known for a long time, and can be traced back to the observation made by Jenner in 1804 that herpes may prevent the development of vaccinia lesions. Interference was first observed as such in observations on plant virus diseases made in 1929, but in the next

few years interference was observed with animal viruses as well. It was thought for a long time that the presence of virus particles inside a cell was necessary for the production of interference, but in 1957 Isaacs and Lindenmann showed that the interfering agent was a substance produced in the cell as a result of the virus infection, which they called interferon. In subsequent work interferon was found to be a protein of molecular weight around 20,000. It has a wide spectrum of antiviral activity and has been subjected to intensive study in the hope of utilizing it as an antiviral agent in man. These hopes have not yet been realized, as the use of interferon as a therapeutic agent has been found to be beset by formidable difficulties. Foremost among these is its species specificity, which restricts its activity to virus infections of the same cell type as that in which it was produced. Thus, interferon produced in chick embryo cells will inhibit virus multiplication in chick cells but is inactive in mouse embryo cells. However, there are exceptions to this general rule, since interferon produced in monkey cells is also active in human cells, and consequently interferon for use in man may be produced in primary cultures of monkey cells.

On a basis of weight, interferon is an antiviral agent of very high activity, but this advantage is more than offset by the difficulty of producing interferon in quantity. Thus, the embryonic fluids from 2000 fertile eggs would be required for the production of a dose of interferon sufficient to treat one human patient. The route of administration also poses difficult problems. In the case of a drug, very little of the dose administered may reach the infected tissue, the remainder of the dose being wasted through poor absorption, rapid inactivation by metabolic systems, and rapid excretion. Similar difficulties would undoubtedly be encountered in the use of interferon in animals or man, with the result that the dose administered would need to be considerably increased.

Some reports have appeared on the use of interferon in virus infections of man. It has been shown to inhibit the multiplication of vaccinia virus in the skin when injected previously into the same site (Scientific Committee on Interferon, 1962), and some effect has been observed in the treatment of vaccinial keratitis. An attempt to protect volunteers against certain upper respiratory viruses by the intranasal instillation of interferon was unsuccessful (Scientific Committee on Interferon, 1965), but some reduction in the proportion of successful infections was observed in similar experiments with influenza virus (Soloviev, 1967). In experiments of this type interferon could be applied topically, but for the majority of virus infections a systemic route of administration would be required. So far,

little success has been achieved by the systemic use of interferon, probably in view of the enormous dose which would be required to produce any effect, a difficulty which could only be overcome by the development of preparations of interferon of exceptionally high activity.

However, there is another way out of the difficulty, since the body can be induced to make its own interferon. Apart from viruses themselves, certain substances have the property of inducing interferon. The first of these to be discovered was statolon, a complex anionic polysaccharide produced by cultures of *Penicillium stoloniferum*. It had a prophylactic effect in mice subsequently infected with Semliki Forest, MM and poliomyelitis viruses. It was also active against Coe (Coxsackie A21) virus in tissue culture, provided that the cultures were exposed to the action of statolon before infection. It was subsequently found that the treatment of tissue cultures with statolon induced them to form an inhibitor, which could be extracted from the cells and used to protect other tissue cultures against virus infections. The properties of this inhibitor agreed with the known properties of interferon.

It was subsequently found that the active material in statolon was a double-stranded RNA derived from a virus which was infecting the mycelium. This discovery led to the trial of a number of double-stranded RNAs formed from synthetic polynucleotides. The one which has found most use is poly I:C, a complex of polyinosinic and polycytidylic acids. It is an effective inducer of interferon in tissue culture, and antiviral effects can be observed in animals, and also in herpetic keratitis in man. However, poly I:C has an undesirably high level of toxicity, and there is some evidence that toxicity, particularly to macrophages, is a necessary concomitant of the ability to cause the induction of interferon. If this is true, it seems unlikely that interferon inducers will find any application in the treatment of virus diseases of man.

Some antiviral agents have failed to fulfil their initial promise. One of these is N^1,N^2-anhydro*bis*(β-hydroxyethyl)biguanide hydrochloride (ABOB), which has been marketed under the names of Flumidin and Virugon. When mice were infected intranasally with a small dose of the PR8 strain of influenza virus and given ABOB by mouth, the extent of the pneumonia which developed as a result of the virus infection was less than in a control group of mice which received the same dose of virus but were not treated with ABOB. Protection was not observed when larger doses of virus were given (Melander, 1960). Some reduction in haemagglutinin production was observed in fertile eggs inoculated with influenza virus and treated with the compound. The degree of protection obtained

was not large, but the compound was nevertheless tried in man. At first favourable results were claimed in the prevention and treatment of influenza, but these effects could not be substantiated in more carefully controlled trials, and in 1967 the Standing Joint Committee on the Classification of Proprietary Preparations in Great Britain placed ABOB in the category of unacceptable preparations which are not of proven value.

Another compound, amantadine hydrochloride, is still under evaluation. This is an amino derivative of the tricylic saturated hydrocarbon adamantane. The hydrochloride will inhibit the multiplication of types A, A1, A2 and C influenza viruses in cultures of chick embryo fibroblasts. A maximum inhibition of 1 log unit was obtained with a concentration of 25 mg/ml (Davies *et al.*, 1964). When mice were infected intranasally with the Swine, WS and Asian strains of influenza virus in small doses (3–4 LD_{50}), some increase in the proportion of surviving animals was noted in comparison with untreated controls. The protective effect was slight, and was considerably reduced when the infecting dose of virus was increased. The mechanism of action was studied in tissue culture, and it was found that amantadine hydrochloride was only effective when it was added to the cultures before they were infected with virus. The compound did not inactivate virus directly, but appeared to inhibit its attachment to the cell surface and subsequent penetration of the cell.

The activity in tissue cultures and in mice was not of a very high order, but the compound has nevertheless been widely tried in man. Jackson, Muldoon and Akers (1963) observed some reduction in the incidence of infection in volunteers who were treated with amantadine and infected with an attenuated strain of Asian influenza virus. The compound was only effective if it was given before infection. Wendel, Snyder and Pell (1966) tried the prophylactic effect of the drug during a natural outbreak of influenza infection; a somewhat lower incidence of infection was noted in persons treated with the drug in comparison with controls treated with a placebo. Other trials have since been reported in which positive results were obtained. However, Tyrrell, Bynoe and Hoorn (1965) investigated the effect of amantadine in a double-blind trial in volunteers infected intranasally with two strains of A2 influenza virus. The result was entirely negative. Amantadine hydrochloride was licensed in 1966 under the Federal Food, Drugs and Cosmetics Act for use in man in the U.S.A., and was shortly after marketed under the name of Symmetrel. The next few years will undoubtedly show what place amantadine has in the treatment or prevention of influenza.

Antiviral chemotherapy is now an established fact, since there are at least two drugs available which are effective in man in preventing or treating certain virus infections. There is also a growing awareness of the fact that antiviral agents may be more effective in preventing virus infections than in treating them. At the same time there are signs that the climate of opinion is moving away from the development of vaccines as the method of choice in overcoming virus infections. The discovery of adventitious agents in virus vaccines requires the application of safety measures which are far more cumbersome and expensive than those which were considered sufficient a decade or more ago. The use of antiviral drugs would not require such extensive control measures and it now remains to discover compounds active against viruses which are still resistant to chemotherapy. Two lines of development can be envisaged here. Firstly, the spectrum of activity of existing drugs may be widened as the result of further study; this has already happened in the case of methisazone, which is active against the unrelated groups of pox viruses and adenoviruses. Secondly, other antiviral agents will no doubt be discovered, and it is still a moot point whether each unrelated group of viruses will require the discovery of an entirely new antiviral agent.

REQUIREMENTS OF ANTIVIRAL AGENTS

The basic property required of an antiviral agent is that it should be effective in treating a virus infection by inhibiting the reduplication of the virus. According to this definition, anti-inflammatory agents which may mitigate the response of the host to the infective process are not to be considered as antiviral agents, although they may be useful adjuncts to treatment.

There are three situations in which a compound can exert antiviral activity; outside the cell, on the cell surface, and inside the cell. A classic example of the first type of action is neutralization by specific antibody. However, neutralizing antibody has proved of little value in the treatment of virus infections, with the exception of infective complications of smallpox vaccination, and its main value is in prophylaxis. This failure to exert an antiviral effect outside the cell offers little hope of success with synthetic compounds acting in the same manner, but in spite of this, promising results have been obtained with certain isoquinoline derivatives which inactivate myxoviruses and picornaviruses (Brammer, McDonald and Tute, 1968).

The second type of action is exemplified by amantadine hydrochloride, which prevents the attachment of influenza virus to cell surfaces. Although compounds with these two types of action may be effective in certain circumstances, they are presumably powerless to arrest virus infections which spread by direct contact between cells, and would not have any effect on viruses which do not appear to require specific receptors for attachment to cell surfaces.

The third type of action is of greater theoretical interest and is incidentally exemplified by antiviral agents which have a much higher activity than the isoquinolines and amantadine hydrochloride. It had long been thought that viruses were invulnerable to attack since they utilized the metabolism of the host in their multiplication, so that anything which affected this metabolism would be toxic. In contrast, the search for antibacterial agents is in principle much more likely to be successful, since bacteria utilize metabolic pathways which are different from those of the host, for example, the folic acid pathway which is sensitive to sulphonamides, and muramic acid, essential for cell wall formation, the production of which is inhibited by penicillin. Bacterial ribosomes are larger than mammalian ribosomes, and antibiotics will inhibit the synthesis of polypeptide chains on them, without having any effect on chain formation in the mammalian host, in which the smaller ribosomes are not affected by the antibiotics.

Viruses, on the other hand, utilize host cell ribosomes for the synthesis of their specific proteins. Some of these proteins are enzymes, however, and the possibility of specificity now arises. Multiplication of the virus involves the replication of its DNA or RNA; this is carried out by respective polymerases which are coded for in the virus genome, and these are antigenically distinct from those present in the host cell.

Agents which interfere with these activities have been found by chance. Thus, 2-(α-hydroxybenzyl)benzimidazole prevents the appearance of the specific RNA polymerase induced in the host cell by the picornaviruses. Guanidine acts at a later stage, and appears to block the initiation of the chains of poliovirus RNA which are synthesized by the virus-specific enzyme. The synthesis of the structural proteins of the virus also appears to offer an opportunity for specific attack, since methisazone causes instability of the messenger RNA of vaccinia virus which carries the code for synthesizing the late proteins of the virus, as a result of which the assembly of infective particles is prevented.

Specificity of attack is also achieved by natural means in the case of interferon. This is a protein which is produced in cells in response to a

virus infection, and also certain other stimuli. Interferon acts by inhibiting the synthesis of virus proteins, and is completely inactive against systems of the host cell.

The multiplication of a virus can be inhibited by substances which lack specificity, and some examples of this class have been discovered in the course of cancer chemotherapy. A number of substances are known which inhibit protein synthesis by interfering with the transcription of DNA or by preventing translation from messenger RNA by the ribosomes. Examples of such substances are actinomycin D, mitomycin C, puromycin and cycloheximide. Since they interfere with the mechanism of the host cell it is not surprising to find that they will also inhibit the multiplication of viruses, by denying the use of the mechanisms required for synthesizing virus material. Since their action is directed against the host cell, such compounds are very toxic, and have therefore not found practical application as antiviral agents in man.

The further outlook for the discovery of specific antiviral agents is not good. The complexity of the systems involved and the inadequacy of our present knowledge of their chemical structure make it difficult or impossible to design compounds with the required action, and it is to be expected that chance will once again play the main part in the next round of discoveries.

The degree of activity required in an antiviral agent before it becomes a candidate for use in man is very high. A compound which will protect against a few LD_{50} of virus in animals or a few ID_{50} in tissue culture is not likely to be of much use, since it can be expected to do little more than prolong the incubation period when used in prophylaxis. Many such compounds have been described in the literature, but very few of them have come to clinical trial, and in no case has any degree of clinical usefulness been demonstrated.

A method of assessing the activity of an antiviral agent in comparison with the maximum activity which is theoretically attainable has been proposed by Bauer (1966). As initial assumptions it is postulated that a compound is maximally effective if 1 molecule can inhibit the maturation of 1 virus particle, and that to attain maximum activity each volume of cytoplasm equal in volume to the volume of a virus particle must contain 1 molecule of compound. From these postulates one can devise a relationship between the minimal inhibiting concentration of a maximally effective compound and the particle radius of the virus against which it is active. It is:

$$\log M = -0.4045 - 3 \log r$$

where M is concentration of the compound as a molarity, and r is the radius of the virus particle in mμ. Figure 1 shows log M plotted against the log of the particle diameter, a more familiar quantity. The points represent values calculated from literature data for minimal effective concentrations of a number of existing antiviral agents. None of the compounds lies between the line and the origin, and the basic assumptions are therefore not invalidated. The vertical distance of a point from the line represents the ratio between the actual minimal effective concentration

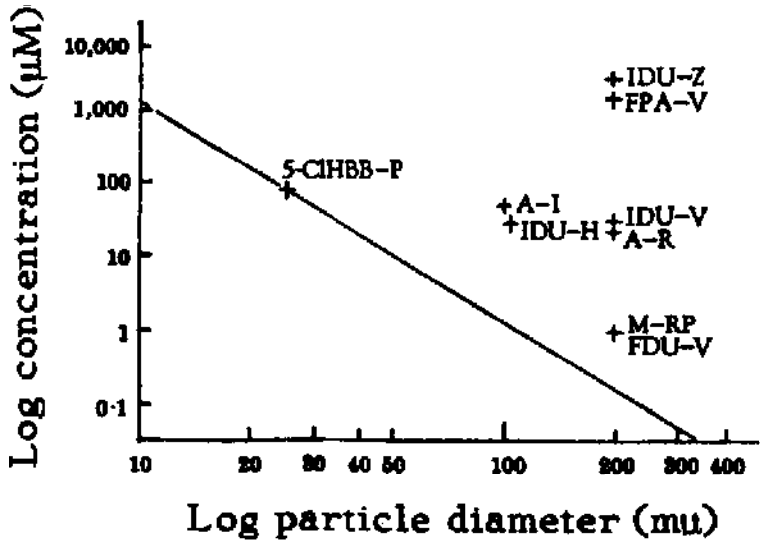

FIG. 1. **Relationship between minimal effective concentration of an antiviral compound and virus particle diameter. The line represents the theoretical relationship for a maximally effective compound. The points represent the activities of known antiviral compounds against the relevant viruses. 5 Cl HBB, 5-chloro-2-(α-hydroxybenzyl)benzimidazole; A, adamantanamine hydrochloride; IDU, idoxuridine; FPA, *p*-fluorophenylalanine; M, methisazone; FDU, 5-fluoro-2′-deoxyuridine; P, poliomyelitis; I, influenza; H, herpes; Z, zoster; V, vaccinia; R, rabbitpox.**

for the compound concerned and the theoretical value for the particular virus. It is therefore a measure of the efficiency of the compound. One point lies on the line (5-chloro-2-(α-hydroxybenzyl)benzimidazole and poliomyelitis); the compound is therefore 100% efficient, and it may be predicted that no more effective compound for poliomyelitis can be found. Similarly, amantadine hydrochloride is only 2.2% efficient against rubella and 2.5% against influenza.

It is possible to predict minimal inhibitory concentrations for viruses for which effective antiviral agents have not yet been discovered, and

these are shown in Table 1. The values have been calculated for compounds with a molecular weight of 200, and the last column shows the equivalent dose in mg for an adult weighing 70 kg. This dose would give effective tissue levels only if it were perfectly absorbed and distributed, and in practice the doses would have to be larger to allow for imperfections in these factors and also for loss by metabolism and excretion. It will be seen that for viruses of particle size ranging down to 45 mμ the revised doses are within the practical range, but that treatment of rhinovirus infections would require doses of several grams, and in the case of the smallest viruses the doses might be impractically large. These theoretical limitations could only be overcome by the discovery of compounds which became selectively concentrated at the site of action, and to be of practical use the compounds would need to be of low molecular weight.

TABLE 1. RELATION BETWEEN PREDICTED MINIMAL INHIBITORY CONCENTRATION OF ANTIVIRAL COMPOUND AND VIRUS PARTICLE DIAMETER

Virus	Diameter (mμ)	Minimal inhibitory concentration (mg/l.)	Equivalent adult dose (mg)
Varicella	200	0.03	2
Mumps	140	0.09	6
Measles	140	0.09	6
Rubella	~130	0.11	8
Respiratory syncytial	130	0.11	8
Influenza	~100	0.25	17
Cytomegalovirus	87	0.37	26
Adenovirus	80	0.38	27
Verruca	45	2.7	190
Rhinovirus	31	8.3	580
Coxsackie	28	11.3	790
Tick-borne encephalitis	25	15.8	1100
Echo	25	15.8	1100
Foot-and-mouth disease	22.5	21.7	—
Infective hepatitis	15	73.3	5100

SCOPE OF ANTIVIRAL CHEMOTHERAPY

The attitude of the medical profession towards the possibility of success in the specific treatment of virus infections has for long been pessimistic. Two characteristic features of virus infections may have been responsible

for this. The course of infection is frequently acute and self-limiting; there is therefore little time available for instituting specific treatment even if it existed. Also, the multiplication of viruses takes place inside the cell and utilizes its synthetic mechanisms; this implies that it is unlikely that this process can be interfered with without producing an unacceptable degree of toxicity to the host.

There are certainly many virus diseases in which the course affords little or no time for specific treatment, such as influenza, haemorrhagic smallpox, rabies, yellow fever, poliomyelitis and infective hepatitis, and in diseases such as these, tissue damage may be irreversible and multiplication of the virus at a maximum by the time that the illness becomes manifest clinically. There are, however, many diseases which have a more protracted course, such as measles, chickenpox and adenovirus infections. Vaccinia gangrenosa may progress for months before the fatal outcome, and infections such as warts and molluscum contagiosum have an even longer course. In another group of infections the disease attracts attention from the very outset by occurring in an external situation, such as infections of the eye due to herpes, vaccinia, zoster and adenoviruses, or infections of the skin such as eczema vaccinatum, vaccinia gangrenosa, or lesions of primary smallpox vaccination which require treatment on account of their severity or ectopic occurrence in unfavourable situations on the face or around the orbit. The disease may have a prodromal phase, as in measles and smallpox, during which it may be diagnosed and treated, or its course may be biphasic, as in echovirus meningitis and infections with viruses of the tick-borne encephalitis complex, with an initial illness followed by a relatively symptom-free period before the more serious phase of multiplication in the central nervous system begins.

The manner in which viruses utilize the synthetic mechanisms of the host cell for the synthesis of their own components is now understood in outline. Enzymes arise in the infected cell which can replicate the virus DNA and RNA, and carry out related functions required for the multiplication of virus material. By immunological methods it has been shown that these enzymes differ from the similar enzymes normally present in the host cell, that is, they are coded for by the virus and are specific to it. This specificity offers a point of attack for chemotherapy, and has been discussed in the preceding section.

Specific treatment cannot be used in a virus infection unless a diagnosis can be made. This may be difficult, since many virus infections present as fevers or upper respiratory infections of unknown origin, which are seldom diagnosed unless they are occurring in epidemic proportions.

Infections of this kind will not be amenable to specific treatment until such time as antiviral agents with a wide spectrum of action become available.

The emphasis so far has been on treatment, but it has become evident during the last few years that the main field of application of antiviral chemotherapy is in chemoprophylaxis. Thus, methisazone will prevent the development of smallpox in persons who have been in contact with a case of the disease and are already in the incubation period of the infection, and it appears that compounds which have a prophylactic effect against influenza are now becoming available.

Chemoprophylaxis offers certain advantages over vaccines as a method of solving outstanding problems in the prevention of virus infections. While it is theoretically possible to make a vaccine against any virus disease, there are often serious difficulties in practice. The rhinoviruses, for example, exist in many serotypes which vary from year to year in prevalence. Some viruses will only grow to low titre, or may be poorly antigenic. Vaccines are produced in living cells which may contain adventitious agents, and stringent safety tests must be applied which render production cumbersome and even uneconomic. Chemoprophylactic drugs are cheaper to produce than vaccines and may be stored for indefinite periods. Their protective effect is immediate and they can therefore be used once an epidemic has broken out, whereas effective use of vaccines requires that the risk of infection shall be correctly assessed in advance. However, chemoprophylaxis suffers from the serious disadvantage that a drug cannot be designed to combat a specific virus infection in the way which is possible with vaccines, and the use of drugs is associated with problems of toxicity and side-effects. Very few drugs are available as yet for the prophylaxis of virus infections, but it is hoped that more will be forthcoming now that the feasibility of chemoprophylaxis has been demonstrated.

DETECTION OF ANTIVIRAL ACTIVITY

Before a test of antiviral activity can be carried out it is necessary to select some system in which replication of the virus gives rise to a visible effect, or an effect which can be measured by some other means. Thus, many viruses produce a cytopathic effect in tissue culture which is evidence of virus multiplication. The effect may consist of cell destruction or production of abnormal forms such as syncytia, and in a number of cases the virus can be induced to form plaques. Viruses of the pox and herpes

group will produce pocks on the chorioallantoic membrane. Infection of animals may give rise to visible lesions, as for example vaccinia on the rabbit skin or cornea, and injection by intracerebral and other routes may give rise to a fatal infection.

Once the system has been selected it can be set up in replicate in the presence and absence of the test compound. If the effect selected as a measure of virus replication is reduced, delayed or completely suppressed, this may be taken as prima facie evidence of antiviral activity. In further studies it should be possible to show that the alteration in effect brought about by the test compound bears some sort of quantitative relationship to the concentration or dose in which the test compound is used, so that a dose–response curve may be obtained for the purpose of assaying the relative potencies of the test compound and its derivatives.

TISSUE CULTURE METHODS

Viruses vary considerably in their effect on cells in culture, and the method selected will depend upon the properties of the virus concerned. It may produce complete destruction of the cell sheet, limited areas of destruction which result in the formation of plaques, or it may cause the appearance of a special function, such as haemagglutination, haemadsorption or neuraminidase activity. The antiviral test selected for use may thus be based on an infectivity end-point titration, or inhibition of plaque formation or special functions.

(i) INFECTIVITY TITRATION

Serial decimal dilutions are made of a suitable preparation of virus and are used to infect two series of tissue cultures in parallel. The cultures may be grown in tubes or small flasks. After allowing a suitable time for infection, which will depend upon the kinetics of adsorption of the virus, the infecting inoculum is removed and replaced with maintenance medium. In one series of dilutions the maintenance medium contains a suitable concentration of the compound to be tested. The cultures are returned to incubation. They are examined at intervals for evidence of cytopathic effect. It is convenient to record the time in days or half-days required for 50% destruction of the cell sheet as estimated subjectively. By averaging this time over a number of replicate tubes any delay in the development of the cytopathic effect in the tubes containing the test compound will become apparent. The final end-point is recorded, and may be lower in

the presence of the test compound. Delay in cytopathic effect and depression of titre both afford evidence of antiviral action.

A disadvantage of the method is the fact that the virus comes into direct contact with the compound in solution when it is liberated from the infected cell at the end of the multiplication cycle. In these circumstances it may be inactivated by chemical means, leading to the erroneous conclusion that the compound has intracellular antiviral activity. It is therefore necessary to set up a control experiment in which a suspension of the virus is mixed with a solution of the compound and incubated at 37°C. Samples are taken at intervals and titrated, and the decline in virus titre should not be greater than that produced by thermal inactivation in the absence of the compound. It is also necessary to show that the compound is being used in a concentration which is not toxic for the cells, since levels verging upon toxicity may depress cell metabolism to such an extent that it is not capable of supporting the synthesis of virus components, in which case a false positive result will be recorded.

(ii) PLAQUE INHIBITION

Many viruses form plaques in suitable cell systems, and inhibition of plaque formation is an effective means of detecting antiviral activity. Suitable test methods were developed independently by Herrmann *et al.* (1960) and Rada *et al.* (1960). A confluent monolayer of a susceptible cell line is grown in a plastic dish and infected with a suspension containing enough virus to produce a large number of plaques. After allowing sufficient time for adsorption of the virus to take place, the monolayer is washed and an overlay of agar is placed on it, containing salts and nutrients appropriate to the cell system used. A filter paper disc impregnated with the test compound is placed on top of the overlay in the centre and the plate is incubated for sufficient time to allow plaques to develop. The test compound diffuses from the disc through the agar, and if it possesses antiviral activity the formation of plaques will be suppressed in an area surrounding the disc over which the compound is present in sufficient concentration. Beyond this area plaques will develop. The plaques are usually rendered visible by pouring on a second overlay containing methyl red or tetrazolium, which stain living cells and show up the plaques by contrast. The general appearance of a typical result with an active compound is shown in Fig. 2. Immediately surrounding the disc the concentration of compound in the agar may reach toxic levels, and the disc is then surrounded by a clear zone of cell lysis. The plaque inhibition method

thus has the advantage that antiviral activity and toxicity can be demonstrated in the same experiment, and it is thus very useful for showing up minor degrees of antiviral activity which might act as leads for further development.

The spread of infection is largely from cell to cell, since spread through the medium is inhibited by the presence of the agar, and the plaque inhibition method is therefore not likely to give false positive results due to inactivation of the virus by direct contact with the test compound. It suffers from the common disadvantage of tissue culture methods, in that compounds of low solubility, although possessing some degree of antiviral activity, may escape detection. The amount of compound in the disc may range up to 100 μg, and unless its solubility in water is sufficiently high it will be necessary to dissolve it in ethanol or other organic solvent and then remove excess solvent by drying in an oven. In doing this it is important to make sure that the compound has not undergone any chemical change, and the advice of an organic chemist should be sought on this point.

The plaque inhibition test can also be used as a rough method of assay, since compounds of equal activity will give zones of protection of the same diameter, provided always that they diffuse at equal rates, and the method can also be used for comparing the activity of a given compound against different viruses.

A more quantitative estimate of the effective inhibitory concentration can be obtained by a modification of the method in which the test compound is dissolved in the overlay medium in a range of concentrations. It is thus possible to determine the minimum concentration which is just effective in suppressing plaque formation. An example of a test of this kind is shown in Fig. 3. A further refinement of this procedure is the gradient plate method developed by Kucera and Herrmann (1966). Monolayers are grown in plastic flasks. After removal of the medium the flasks are tilted in their long axes and agar overlay medium is poured on. When this has set, the flasks are returned to the horizontal position, a second overlay containing the test compound is poured on, and after setting the incubation is continued as usual. The concentration of compound available to the monolayer at any particular point in the flask is proportional to the ratio of the thicknesses of the two wedge-shaped layers of plain and compound-containing medium lying above it. This can be calculated from the position along the flask at which plaques first begin to appear, thus giving the minimal inhibitory concentration from a single culture.

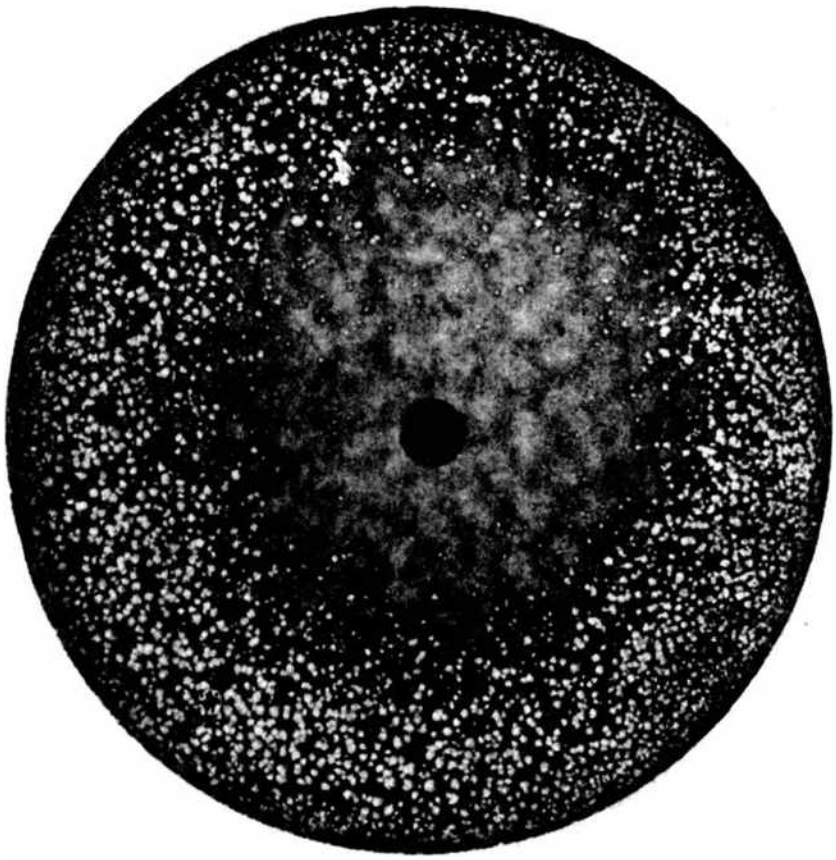

FIG. 2. Plaque-inhibition test. The central disc contains a compound having activity against rabbitpox virus. The compound has diffused through the agar overlay and the disc is surrounded by a zone in which the formation of plaques has been inhibited.

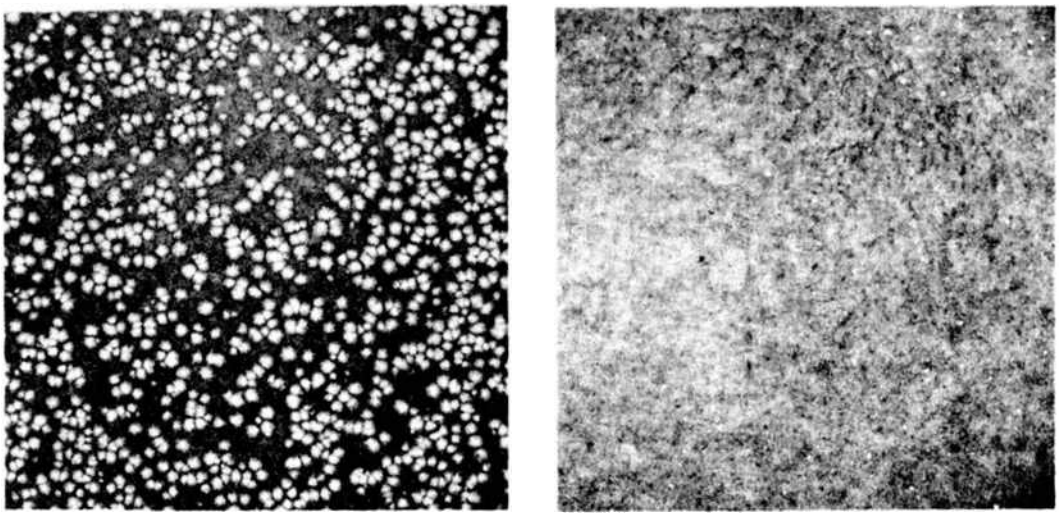

FIG. 3. Plaque-reduction test. *a*, monolayer of HeLa cells infected with rabbitpox virus covered with an overlay of plain agar; *b*, overlay containing 10 μM methisazone; the plaques are almost completely suppressed.

Methods involving the incorporation of a compound in the overlay medium are not suitable for testing compounds of low solubility, but are not likely to give false positive results, since toxicity is readily apparent.

(iii) METHODS BASED ON SPECIAL FUNCTIONS OF THE VIRUS

A number of viruses do not produce plaques or cytopathic effects readily, but may be quantitated by reason of possessing special properties which can form the basis of titration methods.

Influenza viruses will agglutinate red cells, and this property can be used for titrating the amount of virus present in allantoic fluid or tissue culture medium, and thus for detecting and assaying antiviral effect. This method was used by Tamm, Folkers and Horsfall (1952) in evaluating the antiviral action of benzimidazole derivatives.

Other viruses, including paramyxoviruses and vaccinia, will bring about a modification in the properties of the cells in which they are growing, as a result of which red cells will adhere to the surface. If such a culture is treated with a suspension of red cells and then washed, the number of red cells left adhering to the culture will be proportional to the number of infected cells it contains, and hence to the extent to which the virus has multiplied in the culture in the presence of the antiviral agent under test. The effect can be quantitated by lysing the red cells with water and determining the amount of haemoglobin in solution by spectrophotometry. This method was proposed as a test system for assaying interferon (Finter, 1964), and was first used with vaccinia virus (Driessen and Greenham, 1959).

Cultures infected with myxoviruses will form the enzyme neuraminidase; in addition to being located on the virion the enzyme is also present in the cell and on its surface, and determination of the neuraminidase activity of such a culture will give a value proportional to the amount of virus present. This method has been used for the evaluation of isoquinoline derivatives with activity against influenza viruses (Brammer, McDonald and Tute, 1968). Determination of neuraminidase activity has been adapted to the auto-analyser (Kendal and Madeley, 1969), which would afford a very convenient method of screening anti-influenza compounds.

The extent of multiplication of a virus in a culture can in most cases be readily demonstrated by immunofluorescence, and although the method is not susceptible to quantitation it is nevertheless useful for studying the effect of an antiviral agent upon the course of a virus infection, in particular for demonstrating visually the stage at which multiplication is blocked,

and it may afford a useful line of attack in the case of viruses which multiply poorly without giving rise to any conspicuous signs of infection.

A method of detecting and measuring antiviral activity which was of general application and not dependent upon any special property of the virus concerned would obviously be of much greater value than the methods described hitherto, and it seems likely that the procedure developed by Miller *et al.* (1970) will fulfil these requirements. This is based upon the fact that all viruses must replicate DNA or RNA during their multiplication cycle. The synthesis of nucleic acids in an infected tissue culture can be followed directly by determining the uptake of tritiated uridine and thymidine. The procedure readily lends itself to automation, and the authors point out that 100 cultures can be processed in 3 man hours and the results can be fed directly into a computer for rapid evaluation.

ANTIVIRAL TESTS IN FERTILE EGGS

Before the development of tissue culture techniques, fertile eggs were used extensively for the cultivation of viruses, and in a number of cases they still afford a cheap and convenient system for testing antiviral activity. Viruses of the pox and herpes groups form pocks on the dropped chorioallantoic membrane, and myxoviruses will multiply in the chorioallantoic or amniotic membranes with subsequent liberation into the fluids.

Compounds to be tested for antiviral activity may be injected into the yolk sac, or if sufficiently soluble in water, they may be injected into the allantoic fluid or dropped on to the chorioallantoic membrane after infection. Evidence of antiviral activity can be obtained by observing a reduction in the number or size of pocks, or a reduction in haemagglutinin titre in the case of myxoviruses. It is of interest to recall that prolongation of survival time of the chick embryo was used by Hamre, Bernstein and Donovick (1950) in detecting the activity of *p*-aminobenzaldehyde thiosemicarbazone, the first antiviral compound to be discovered.

Multiplication of the influenza viruses takes place in the chorioallantoic membrane, and the presence of the embryo is not essential. The contents of the egg may therefore be removed, leaving only the chorioallantoic membrane attached to the inside surface of the shell. The membrane is then infected with virus and washed, and the shell is filled with saline and incubated at 37°C. After some hours sufficient multiplication of virus has taken place to give a detectable titre of haemagglutinin in the saline solution. This preparation is known as the de-embryonated egg,

and was first developed by Bernkopf (1949). Compounds to be tested for antiviral activity may be added to the saline solution, and a positive result will give rise to a depression in haemagglutinin titre in comparison with untreated controls. The method has been used extensively in the chemotherapy of the influenza viruses, but suffers from the disadvantage that it is unsuitable for compounds of low solubility, and may give false positive results from inactivation of the virus by direct contact with the test compound in solution. Toxicity is also difficult to detect.

The capacity of the method may be increased by cutting the shell into small pieces with membrane attached. These are infected and incubated in individual containers, thus making it possible for a number of compounds to be tested simultaneously against the same controls.

ANTIVIRAL TESTS IN ANIMALS

Tests in animals were used extensively in the early days of antiviral chemotherapy, and they still form an important intermediate stage between detection of activity in tissue culture or eggs, and consideration for use in man. They are less used at present, since convenient model infections in animals are not available for many viruses of clinical importance.

A typical test would consist of the inoculation of two groups of animals with a known and usually rather small dose of virus. The route of inoculation chosen depends upon the properties of the virus concerned. One group is then dosed with the test compound and the other constitutes a control. The responses in the two groups are then compared.

It is essential the response should be proportional in some way to the dose of virus. In the case of certain neurotropic viruses adapted to passage in mouse brain, Bauer (1960) has shown that there is a linear relation between the reciprocal mean survival time and the log dose of virus used for infection. A reduction in the former quantity indicates an effective reduction in the infecting dose, and thus an antiviral effect. With a number of other viruses, mainly arboviruses and neurotropic influenza viruses, there is no such relation, and the reciprocal mean survival time may remain essentially constant when the infecting dose of virus is varied over a 1000-fold range. In a system such as this, an antiviral effect would remain undetected.

Examples of the types of antiviral tests which may be carried out in animals will be found in the following chapters. It is of historic interest to note that the antiviral properties of methisazone and idoxuridine were first found by animal experiments.

MEASUREMENT OF ANTIVIRAL ACTIVITY

Once antiviral activity has been detected it is necessary to quantitate it, so that the effects of modifications of the molecule may be properly assessed. It is first necessary to select some response to infection which is proportional to the concentration of compound in tissue culture, or the dose administered to animals. The type of response chosen may be quantal or continuous.

A typical quantal response would be the LD_{50}, as determined by titrations in animals, in which the response is based upon whole numbers of animals dying or surviving. The same applies to the ID_{50} for eggs or tissue cultures, and to haemagglutinin and other titrations. Quantal methods lack sensitivity, since each experimental unit can only provide two pieces of information. An animal either dies or survives, and a tissue culture tube either develops the virus infection or escapes it.

When quantal methods are selected for antiviral testing, the titration end-points are determined, usually roughly by the method of Reed and Muench (1938), and a simple test of significance is applied to any difference in titre between test and control series. This method is simple and convenient for the initial detection of antiviral activity, but it is not suitable for the development of methods of assaying the relative potency of analogues of the compound first discovered.

A continuous response method was first used by Gard (1940). He observed that the reciprocal of the incubation period in mice infected with the GD VII and FA strains of mouse encephalomyelitis virus was linearly related to the logarithm of the dose of virus used for infection. The mean reciprocal incubation period could thus be used to titrate the virus content of a virus preparation by interpolation from a previously determined response curve. The method was far less cumbersome than standard titration procedures, but it failed to come into general use.

The same relation was found to hold for the reciprocal survival time of chick embryos infected with vaccinia virus in the yolk sac (Brownlee and Hamre, (1951), who used this principle as a basis for the development of a test of antiviral activity). Gordon Smith and Westgarth (1955) showed that it could be used for the determination of neutralizing antibody. The linear relation between reciprocal survival time and virus dose was found to hold for a number of neurotropic virus infections in mice (Bauer, 1960), and the departure from linearity at low virus doses observed by Brownlee and Hamre (1951) due to the occurrence of survivors could be

obviated by the device of counting the survival time as infinity. This gives a reciprocal of zero, and if the zeros are added to the non-zero reciprocals before taking the mean, linearity was still preserved.

The effect of an active antiviral compound is to prolong survival time, and this is equivalent in effect to a reduction in the infecting dose of virus. Since the mean reciprocal survival time is linearly related to the logarithm of the virus dose, it is thus reasonable to expect that it should also be related in some way to the dose of compound administered. This was found to be so by Bauer and Sadler (1960) for mice infected with vaccinia virus and treated with compounds of the isatin 3-thiosemicarbazone series.

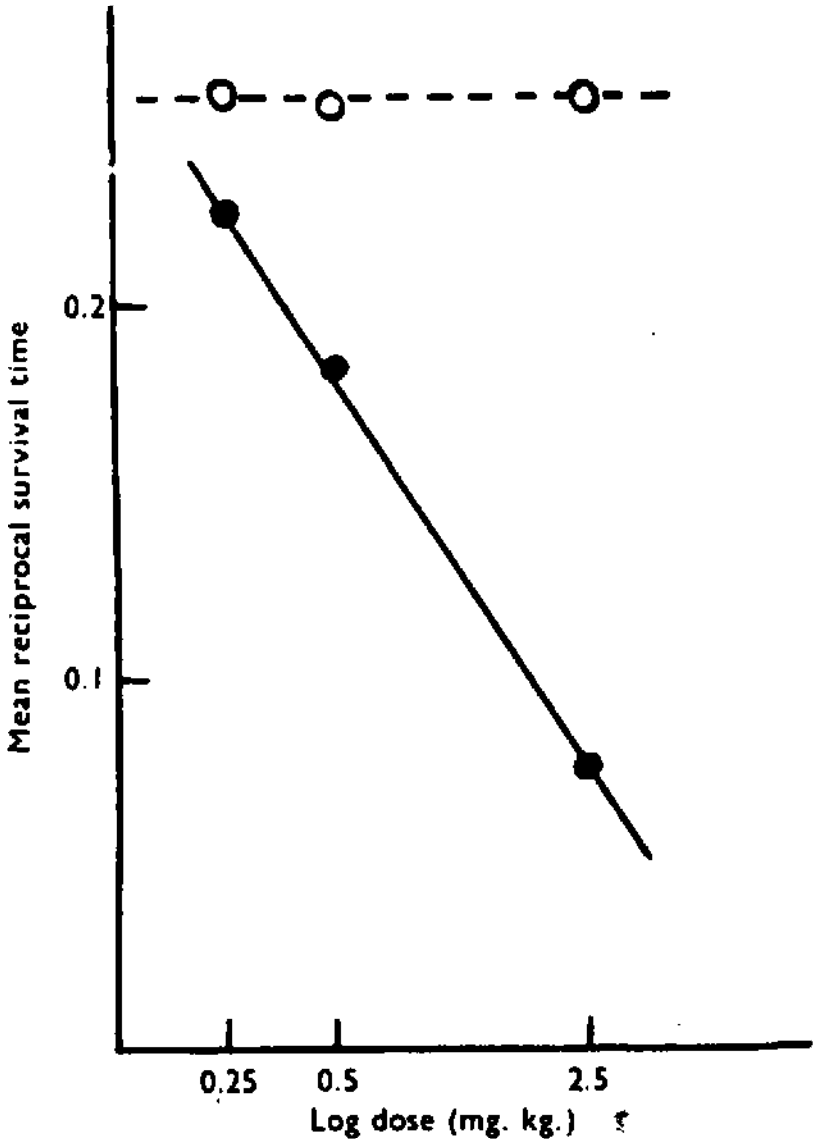

FIG. 4. Dose–response line of 1-propylisatin 3-thiosemicarbazone against neurovaccinia virus. Mice were infected with the virus intracerebrally and injected with the compound subcutaneously in the doses shown. The ordinate represents the mean reciprocal survival time (Bauer and Sadler, 1960).

The mean reciprocal survival time had a linear regression on the logarithm of the dose of compound. A dose–response line for 1-propylisatin 3-thiosemicarbazone obtained by this method is shown in Fig. 4. Once a system which yields dose-response lines has been devised, it is possible to evaluate the relative potencies of derivatives by carrying out a 4-point or 6-point assay against a standard, which is normally the parent compound. An example of a 4-point assay of 7-methylisatin 3-thiosemicarbazone against isatin 3-thiosemicarbazone is shown in Fig. 5. The relative potency is indicated by the horizontal distance between the two lines, which represent the best fit for the pooled data obtained by the least squares method. Making allowance for the differences in dose levels, the relative potency R is given by

$$\log R = \log x_S - \log x_T - (\bar{y}_S - \bar{y}_T)b^{-1}$$

where x_S is the mean dose of the reference compound (isatin 3-thiosemi-

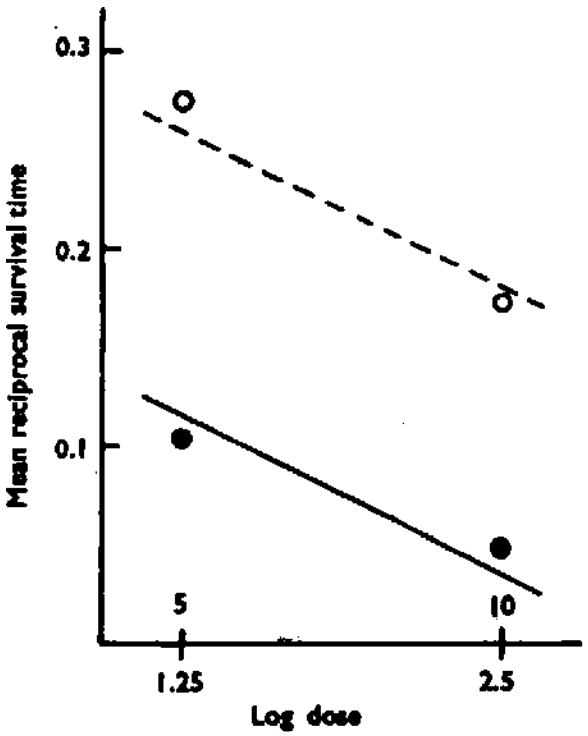

FIG. 5. Four-point assay of activity of 7-methylisatin 3-thiosemicarbazone against the standard compound isatin 3-thiosemicarbazone. Mice were infected intracerebrally with neurovaccinia virus and injected subcutaneously with the compounds in the doses indicated. ●, Isatin 3-thiosemicarbazone, doses 1.25 and 2.5 mg; ○, 7-methylisatin 3-thiosemicarbazone, doses 5 and 10 mg. The ordinate represents the reciprocal mean survival time (Bauer and Sadler, 1960).

carbazone), x_T the mean dose of the test compound, y_S and y_T the mean responses, and b the slope of the regression lines.

The dose–response line can also be used as a means of determining potency in absolute terms without reference to a standard. The mean reciprocal survival time in a control group of untreated animals infected with the same dose is determined. The dose of test compound corresponding to this value on the dose–response line represents the dose which is just insufficient to produce any effect. This is the E_0, or zero effect dose (Bauer, 1961). In the case of mice infected intracerebrally with vaccinia virus, E_0 is independent of the dose of virus used for infection over a range

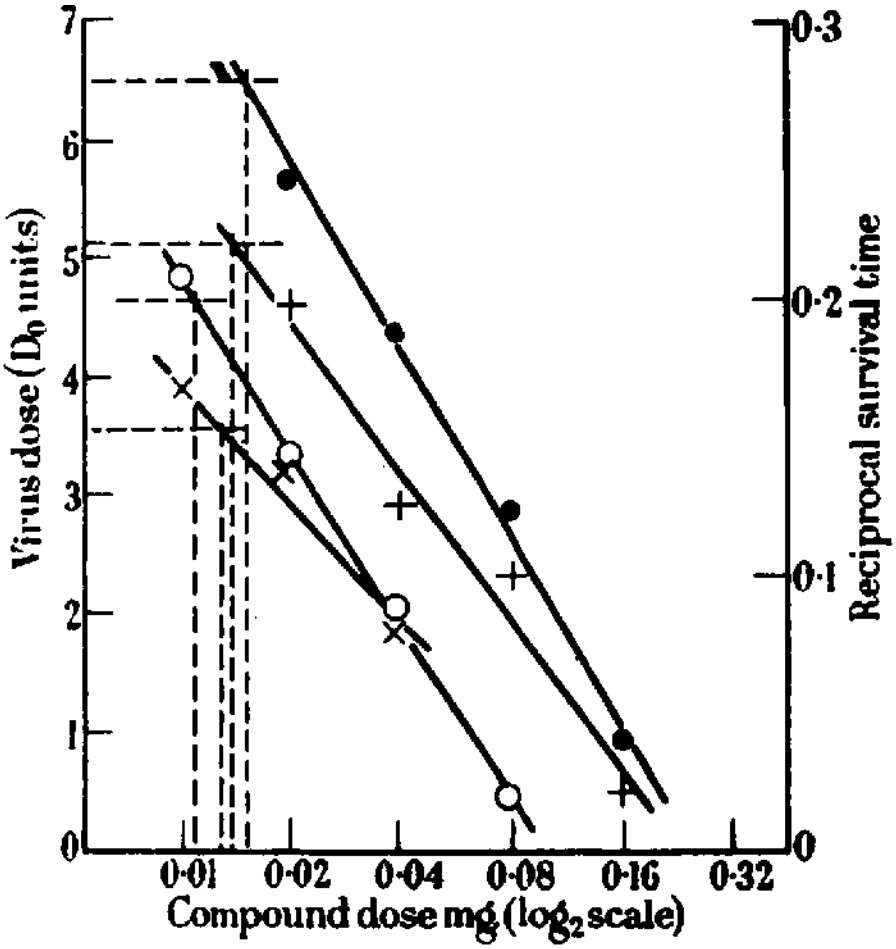

FIG. 6. Dose–response lines of the antiviral action of isatin 3-thiosemicarbazone against varying doses of neurovaccinia. Mice were infected intracerebrally with decimal dilutions of the virus and treated with the compound in the doses indicated. *Ordinates:* left, $\log_{10}$ dose of virus in D_0 units, right, mean reciprocal survival time; *abscissa:* dose of compound on $\log_2$ scale. The horizontal dotted lines indicate the mean value of the ordinates obtained in the absence of treatment. The point of intersection with the dose–response lines occurs at a dose (E_0) which is essentially constant whatever the dose of virus (Bauer, 1961).

of 3 log units (Fig. 6). It can therefore be used as an absolute measure of antiviral activity, since it obviates the necessity of using a standard compound for the purpose of comparison.

Other absolute methods of measuring activity include the ED_{50}, the dose or concentration which produces an antiviral effect amounting to 50% protection, and the ED_{75}, which has been used for evaluating the antiviral activity of benzimidazole derivatives against influenza (Tamm *et al.*, 1953).

A continuous response is also obtained from plaque inhibition tests when the diameter of the zone of protection is taken as the measure of activity. The method is not particularly accurate, since the diameter of the zone is dependent on water solubility, rate of diffusion and rate of inactivation of the compound during incubation. It also does not give any information concerning the concentration of compound in the overlay medium. This can be obtained by the gradient plate technique of Kucera and Herrmann (1966) described in an earlier section.

SPECIAL INVESTIGATIONS OF ANTIVIRAL ACTIVITY

Once antiviral activity has been discovered in a group of compounds it becomes of interest to investigate the mode of action. If the activity has been discovered by a tissue culture method it is first necessary to establish whether the virus is inactivated in an extracellular position. This is done by incubating a suspension of the virus with a solution of the compound and determining the rate of loss of infectivity by titrations of samples removed at intervals. If the rate exceeds that in a control preparation incubated similarly in the absence of the compound, it is evident that the compound is inactivating the virus before the latter has entered the cell. Compounds with this site of action are unlikely to be active in animal tests or be of value in man.

The compound may exert its antiviral activity by preventing the attachment of the virus to the cell, by attaching to receptors or other similar mechanism. This can be investigated by determining the rate of uptake of virus by a cell monolayer by titrations of samples of supernatant removed at intervals. The experiment is carried out with medium containing the test compound in solution, having previously established that inactivation does not occur, and with plain medium. Comparison of the absorption curves will show whether uptake of virus has been blocked or not.

If the compound does not inactivate the virus extracellularly and does not block uptake, it may be assumed that the antiviral action takes place

inside the cell. In this case it is first necessary to determine the time during the virus multiplication cycle when the compound exerts its action. It is necessary to infect monolayers with a high multiplicity of virus in order to ensure infection of most of the cells. The content of virus in the culture is then representative of a single cycle of infection. Medium containing the active compound in a concentration known to be sufficient to suppress virus multiplication may then be added at various times after infection and the effect on virus yield is determined. If the compound is no longer active when added in the early or middle stages of the growth cycle it may be inferred that it is inhibiting the synthesis of virus RNA or DNA, or of virus-coded RNA or DNA polymerases. If the compound is still active when added at a late stage in the growth cycle it may be inferred that it is inhibiting the synthesis of virus-specific proteins, and is not affecting nucleic acid synthesis.

Further information may be obtained by adding the compound to the cultures immediately after infection, removing it at increasing intervals and determining the final yield of virus. In some cases, as with rifampicin, the virus will multiply again after the compound is removed, and the final yield will not be greatly affected. If the compound is acting at an early stage in the growth cycle, the multiplication of the virus will still be inhibited if it is removed after an hour or so.

The effect of an antiviral compound on the synthesis of virus-coded proteins can be determined by immunodiffusion experiments. If an extract of infected tissue cells is reacted against an immune serum by the standard Ouchterlony technique, a series of lines will be formed which correspond to the specific antigens of the virus. If the compound acts by inhibiting protein synthesis, some of these lines will be absent. In this manner Appleyard *et al.* (1965) showed that the proteins which appear late in the multiplication cycle of rabbitpox virus are absent in infected cultures treated with isatin 3-thiosemicarbazone.

Much more detailed information concerning the mechanism of antiviral action can be obtained by utilizing the methods of molecular biology. The formation of virus RNA can be followed by adding labelled uridine and determining the radioactivity of RNA extracted by standard methods from cultures sampled at successive intervals of time. RNA synthesis by the host cell is suppressed by incorporating actinomycin D into the medium. If the amount of radioactivity incorporated is reduced or suppressed in the presence of an antiviral compound, it is clearly exerting its effect by inhibiting the synthesis of virus RNA. This method has been developed in detail by Miller *et al.* (1970) as a method of screening antiviral activity.

Synthesis of virus DNA can be followed by determining the uptake of labelled thymidine; extracts of the cultures are incubated with DNAse, and the radioactivity incorporated then represents virus DNA coated with protein which is thus inaccessible to DNAse treatment. Miller *et al.* (1970) have developed the method into a semi-automated procedure in which 400 RNA determinations can be carried out within a period of 24 hr and fed into a computer for evaluation.

CLINICAL TRIALS OF ANTIVIRAL DRUGS

The compound selected for clinical trial may not necessarily be the most active of the series. Other factors come into play, such as cheapness and ease of manufacture on a commercial scale. The compound must also have favourable pharmacodynamic and pharmacokinetic properties, and must have come successfully through its toxicity trials. It is also necessary to develop a suitable formulation and presentation, which will in turn depend upon the route of administration which is selected. The pure compound itself will generally be quite unsuitable as a medicament, and will need to be mixed with other materials for the production of tablets, ointments, solutions for injection, eyedrops and other types of presentation. These points are common to the pharmaceutical development of any drug for clinical use, and do not present any unusual aspects in the case of antiviral drugs.

Once the formulation and presentation have been decided, the drug must next be subjected to clinical trials. These differ in many ways from clinical trials of pharmacological drugs, since the drug is being used against an infectious disease. Thus, trials may be carried out in volunteers, who are infected experimentally with the virus concerned, or the drug may be tried out during a naturally occurring epidemic of the disease. In either case the drug can be given prophylactically or therapeutically.

PROPHYLACTIC TRIALS

If the virus infection concerned is relatively mild, a prophylactic trial can be conveniently carried out in volunteers. As the number of subjects will necessarily be small, it is important to avoid bias in assessing the results. This is particularly so in prophylactic trials against respiratory virus infections, where the criteria of illness which are used for assessing the results are often subjective. The patient and evaluating physician should therefore be unaware of whether drug or placebo is being given, but it is

not necessary to resort to the complication of coding so long as the treatment is allocated by a person who is not participating in the trial.

Probably more important than personal bias is the bias which is introduced by previous immunity. It is important to take pre-infection blood samples for antibody titration, and volunteers already possessing antibody should be excluded. Further specimens should be taken immediately before the beginning of the trial, and any volunteers who have developed antibody in the meantime should be excluded retrospectively. In some cases the existence of immunity can be ascertained without performing antibody determinations. The infectious exanthemata such as measles and chickenpox can be diagnosed clinically with reasonable certainty, and persons admitting to having had these diseases can be excluded on the basis of history alone. In prophylactic trials against smallpox some idea of the state of immunity can be gained by examining for the presence of the scars of primary and subsequent vaccinations.

An important advantage in volunteer trials is that all the subjects are infected with the same dose of virus. There is thus no variation in the degree of exposure, such as occurs naturally in epidemic situations.

The above principles are exemplified in a trial of amantadine hydrochloride against A2 influenza infection carried out by Bloomfield *et al.* (1970). As an additional precaution the volunteers were matched in pairs with respect to age and weight. The latter point is important, since the volunteers will not receive the same dose of drug in mg/kg if they differ much in weight. A statistically significant result was obtained, although only 18 volunteers were used in the trial. A similar method was used by Tyrrell *et al.* (1965), except that volunteers with pre-existing antibody were used, as it was considered unethical to infect persons who were fully susceptible.

If a satisfactory result has been obtained in a volunteer trial the next stage is to try the compound in an epidemic situation. The method employed will depend upon the nature of the disease. If infection is by case-to-case contact and the disease can be diagnosed clinically, the prophylactic trial can be restricted to the close contacts of the patient. This situation exists in the acute exanthematous fevers, such as smallpox, chickenpox and measles, and to a lesser extent in influenza, in which the clinical diagnosis is not quite so reliable. Prophylactic trials of methisazone and other compounds against smallpox have been reported by Bauer *et al.* (1963, 1969) and Rao *et al.* (1966, 1969a), and against alastrim by Ribeiro do Valle *et al.* (1965). The drugs were administered to healthy family contacts of smallpox patients, and the occurrence of secondary cases of

smallpox was recorded during an observation period of 2 weeks or so, which was sufficiently long to cover the usual incubation period of 12 days. A reduction in the incidence of secondary cases in treated contacts in comparison with a similar control group of contacts can be taken as evidence of prophylactic effect, provided that treatment and control groups are equal in respect of the extent of pre-existing immunity, degree of exposure to infection and various demographic factors such as age and sex distribution.

Galbraith *et al.* (1969) carried out a similar prophylactic trial among family contacts of patients suffering from A2 influenza. Treatment with amantadine hydrochloride or a placebo was given for a period of 10 days, and secondary cases of influenza were recorded. The diagnosis was confirmed by serological methods.

In some virus diseases case contact is not so apparent, particularly when an epidemic is occurring in a closed community. In these circumstances a prophylactic trial may be carried out by administering drug and placebo over a prolonged period and awaiting events. The drug must necessarily be of acceptably low toxicity, in view of the prolonged administration, and the treatment and placebo groups must be of considerable size, since the incidence of contact cases may be low. It may be necessary to eliminate a number of contacts on account of pre-existing immunity, especially if the epidemic is already under way, and some contact cases may have to be excluded retrospectively if the diagnosis cannot be confirmed serologically.

Oker-Blom *et al.* (1970) investigated the effect of amantadine hydrochloride in an open community of medical students during an epidemic of A2/Hong Kong influenza in Helsinki in 1969, and a similar study was carried out by Keating (1966) in a closed community of prisoners who were evenly matched for antibody status. Drug and placebo were given for 6 weeks, and an epidemic of influenza occurred during this period. Trials of this type provide information as to the prophylactic effect of a drug when used under natural conditions, but they have the disadvantage that large numbers of subjects must be brought into the trial in order to make sure of having a sufficient number of contact cases in the control group to afford a valid comparison.

THERAPEUTIC TRIALS

A double-blind study with randomization of drug and placebo springs to mind as the correct way of carrying out a trial of therapeutic activity

against virus infections, but in practice the position is complicated by ethical considerations. The therapeutic activities of methisazone and idoxuridine were first demonstrated in uncontrolled trials, in which the assessment of response to treatment was based on clinical impression. Once activity has been demonstrated, it is hardly justifiable to attempt to confirm it by a controlled trial with a placebo, since patients receiving placebo are being denied a treatment which has been assessed as specific and effective. In the case of these two drugs the conditions against which they are used are life-threatening (eczema vaccinatum and vaccinia gangrenosa) or liable to cause serious disablement (herpetic keratitis). Idoxuridine has been used in herpetic encephalitis, a condition which has a high mortality, and will doubtless continue to be used in the absence of any evidence from controlled trials until some more effective compound comes along.

In diseases in which there is no specific treatment such ethical considerations do not arise, and a double-blind controlled trial is indicated, provided that the disease is not so uncommon that an adequate number of cases cannot be accumulated.

Once a person has developed a disease it may be thought that the question of pre-existing immunity is not important. This is not so, since it may have a marked effect upon the severity of the disease and its outcome. Thus, smallpox has a severe course and high mortality in persons who have never been vaccinated, and is considerably milder in persons who have been vaccinated in the past. Rao *et al.* (1969b), in trials of CG662 and methisazone in the treatment of smallpox, subdivided their patients according to vaccination status and type of disease in their assessment of the results of treatment. The time after the onset of illness at which treatment is begun is also of importance, since little effect can be expected if the phase of virus multiplication is coming to an end.

The criteria selected for recording the effect of treatment will depend upon the symptoms and clinical course of the disease concerned. In a trial of the therapeutic effect of M and B 7714 against smallpox Rao, *et al.* (1965) recorded mortality, time to scabbing of the lesions, the mean number of febrile days, the number of afebrile cases after the 4th day of treatment, and the mean maximum temperature. In herpetic infection of the cornea the effect of treatment can be assessed from the mean time of healing of the ulcers.

Hornick *et al.* (1969) investigated the therapeutic effect of amantadine hydrochloride in American prisoners suffering from A2 influenza. A placebo was used and treatment was assigned by double-blind methods.

The temperature was recorded every 4 hr and the presence of the characteristic signs and symptoms of influenza was recorded twice daily. The patients were subsequently subdivided into rapid, medium and slow resolvers, according to whether the temperature fell to 100°F or less within 24 hr, 24–36 hr or longer than 36 hr, combined with a 50% reduction of symptoms.

The criteria used for assessing the effect of treatment will depend upon the nature of the disease. Owing to the scarcity of effective antiviral agents, few trials have been carried out as yet, and the number of diseases which have been treated is correspondingly limited, but some general principles have already emerged. The protocol should list the signs and symptoms which are to be evaluated, and their intensity should be recorded numerically, together with their duration. The resulting numerical data then form the output of the trial, and are then subjected to any statistical treatment which is considered appropriate.

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CHAPTER 1

THIOSEMICARBAZONES

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INTRODUCTION

EARLY HISTORY OF THE THIOSEMICARBAZONES

ALDEHYDES (Fig. 1, I) and ketones (Fig. 1, II) will condense with thiosemicarbazide (Fig. 1, III) under appropriate conditions to form thiosemicarbazones (Fig. 1, IV).

Thiosemicarbazide was first synthesized in 1895 by Freund and Imgart. Its reactions were described in 1902 by Freund and Schander, who proposed the use of thiosemicarbazide as a reagent for aldehydes and ketones in view of the low solubility of the thiosemicarbazones, which usually precipitate from the reaction mixture. The same authors were the first to publish a synthesis of benzaldehyde thiosemicarbazone (Fig. 1, V).

Realization of the potentialities of thiosemicarbazones as chemotherapeutic agents was delayed for another 44 years. Following upon the discovery of the antibacterial action of the sulphonamides, Behnisch was synthesizing sulphathiadiazoles as potential antibacterial agents. Essential intermediates in this preparation were 2-aminothiadiazoles, which were formed from the corresponding thiosemicarbazones by oxidative ring closure with ferric chloride. One of these, benzaldehyde thiosemicarbazone, was sent to Domagk, apparently by chance, to be examined for *in vitro* activity against *Mycobacterium tuberculosis*. It was found to be highly active, and antibacterial chemotherapy had taken another step forward by the discovery of a new class of chemical compounds active against the tubercle bacillus (Domagk *et al.*, 1946). The same workers gave a brief account of the structure–activity relationship of the activity against

tuberculosis. Active thiosemicarbazones were only obtained from cyclic aldehydes or ketones. The sulphur atom in the side-chain was essential for activity, since the corresponding semicarbazones, in which sulphur is replaced by oxygen, had very little activity. Substitution in the *para*-position of benzaldehyde thiosemicarbazone gave compounds of high activity, and also some substitutions in the other two positions. As substituents nitro, amino, alkylamino, acylamino, arylideneamino, alkyl, halogen, cyano, carbonyl, hydroxyl, alkoxy, alkylmercapto, alkylsulphoxide and alkylsulphone groups all gave derivatives with activity.

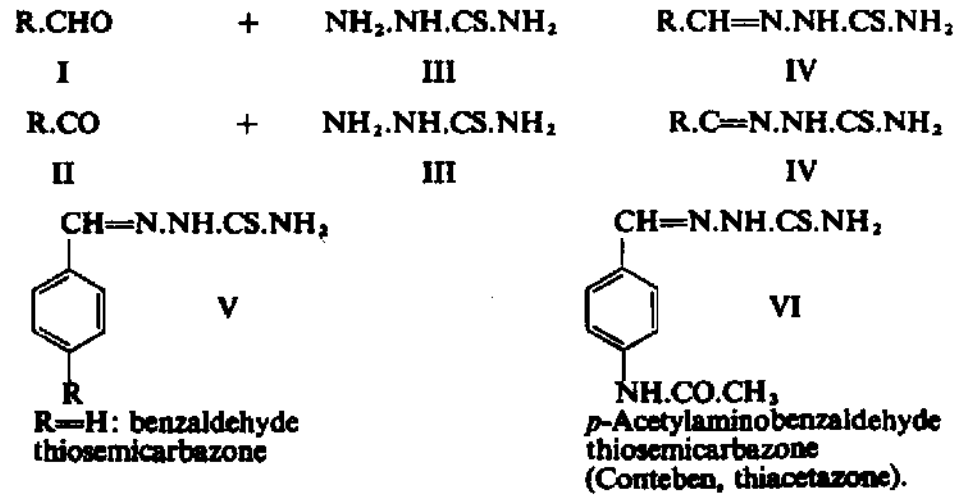


FIG. 1. Structural formulae of thiosemicarbazones. I, an aldehyde. II, a ketone. III, thiosemicarbazide. IV, a thiosemicarbazone. V, a benzaldehyde thiosemicarbazone. VI, *p*-acetylaminobenzaldehyde thiosemicarbazone.

Particularly good results were obtained with *p*-acetylaminobenzaldehyde thiosemicarbazone (Fig. 1, VI), and this was subjected to clinical trial under the names of Conteban and thiacetazone. Favourable results were obtained on 2000 patients with tuberculosis in West Germany, but further development of the drug was limited by the introduction of isoniazid. However, it is still widely used in tropical countries in combination with isoniazid in view of its cheapness.

BENZALDEHYDE THIOSEMICARBAZONES AS ANTIVIRAL AGENTS

The discovery and development of the thiosemicarbazones as agents for the treatment of tuberculosis occurred during a period which saw the first beginnings of work on antiviral chemotherapy. In preparation for

the discovery of active compounds much effort was devoted to evolving reliable test methods, and it must be regarded as a fortunate coincidence that a test system against vaccinia virus had been devised at precisely the time that compounds with antivaccinial activity, namely benzaldehyde thiosemicarbazone and its derivatives, were attracting so much attention in the field of antibacterial chemotherapy.

The test devised by Brownlee and Hamre (1951) was based upon infection of fertile eggs with vaccinia virus. A high-titre pool of virus was prepared from chorioallantoic membranes infected with the New York Board of Health strain of vaccinia virus. On titration in the yolk sac it had a titre of 7.55 log units. Dilutions ranging from 10^{-4} to $10^{-6.5}$ were inoculated into the yolk sacs of groups of fertile eggs at the 6th day of incubation; the eggs were examined at intervals after infection and the number of embryos which had died at each inspection was recorded, giving an approximate value for the survival time. The mean of the reciprocals of the survival times for each group of eggs gave a linear regression over a considerable range when plotted against the logarithm of the dose of virus used for infection. From this it was postulated that if the eggs were treated with an effective antiviral compound which prolonged the survival time, the effect would be equivalent to a reduction in the infecting dose of virus and would manifest itself as a displacement of the regression line by an amount M which would afford a measure of the degree of antiviral activity. The results of 12 experiments carried out with *p*-aminobenzaldehyde thiosemicarbazone given in a dose of 0.01 mg were presented. In each case the treatment produced a displacement of the regression line in comparison with that of untreated controls; the value of $\log_2 M$ thus obtained ranged from 1.49 to 2.66, with a mean value of 1.96. The treatment thus exerted an antiviral effect equivalent to a suppression of 75% of the infecting dose of virus.

Further results with *p*-aminobenzaldehyde thiosemicarbazone were reported separately (Hamre, Bernstein and Donovick, 1950). In groups of 6 fertile eggs infected *via* the yolk sac with 340 LD_{50} of vaccinia virus and given 0.06 mg of the compound by the same route 30 min later, the mean survival time of the embryos (not the mean reciprocal) was 128 hr, compared with 96 hr for a control group treated with saline. With an infecting dose of 170 LD_{50} the mean survival time was 151 hr compared with 119 hr for the controls. With both dose levels of virus all the treated eggs died. When the dose of virus was reduced to 85 LD_{50}, 3 of 6 treated eggs survived, and the mean survival time was 191 hr, compared with 109 hr in untreated controls, which all died. Further experiments were

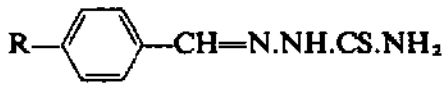
carried out, in which an antiviral effect was also obtained. In one experiment with the related compound *p*-acetylaminobenzaldehyde thiosemicarbazone in a dose of 0.01 mg, the mean survival time was 158 hr, compared with 98 hr in the controls. The compound could not be tested in higher doses on account of its toxicity.

Some antiviral activity was also detected with *p*-aminobenzaldehyde thiosemicarbazone in 10 mice infected intranasally with 0.5×10^6 pock-forming units of vaccinia virus. The compound was given daily for 4 days by subcutaneous injection. All the mice died, but the mean survival time was 8.0 days, compared with 6.2 days in a control group of infected animals injected with saline. The antiviral activity observed in eggs was thus confirmed in animals. It was further observed that the compound was active when given by mouth, and that it had no detectable activity in mice infected intranasally with the Shope strain of swine influenza virus.

The structure–activity relationships of the substituted benzaldehyde thiosemicarbazones were studied by Hamre, Brownlee and Donovick (1951). Tests were carried out in eggs infected with vaccinia virus *via* the yolk sac according to the method described previously. The benzene ring was found to be essential for activity, since the analogues derived from glucose and cyclohexane had no effect. The semicarbazones, which have oxygen instead of sulphur in the side-chain, were inactive, and also thiosemicarbazide. Antiviral activity thus required the presence of the benzene ring and the side-chain containing sulphur. Antiviral activity did not parallel activity against *Myco. tuberculosis*. Thirteen mono- or disubstituted benzaldehyde thiosemicarbazones were tested in eggs, and the antiviral activity was expressed as the equivalent reduction in the infecting dose of virus. The compounds were tested at varying dose levels related to the maximum tolerated dose. The greatest antiviral effect was obtained with 4-methoxybenzaldehyde thiosemicarbazone given in a dose of 0.125 mg, which produced an equivalent reduction of 3 log units, compared with 2 log units for the parent compound in the same doses. Reductions of 2 log units or more were also obtained with the 4-amino, 4-ethylsulphonyl and 4-propoxy derivatives. The 4-hydroxy-3-methoxy and 4-hydroxy-3-sulphonyl derivatives had no significant activity. Conditions in this preliminary survey were too variable to enable the respective activities to be adequately compared, and in further work 6 of the more active 4-substituted derivatives were compared against the parent compound in a constant dose of 0.006 mg per egg. The results are shown in Table 1.

The significance of the differences in activity observed was not reported,

TABLE 1. STRUCTURE–ACTIVITY RELATIONSHIPS OF 4-SUBSTITUTED BENZALDEHYDE THIOSEMICARBAZONES AGAINST VACCINIA VIRUS IN EGGS. From Hamre *et al.* (1951)



R	Activity†
—	1.34
Amino	1.62
Methoxy	1.98
Acetylamino	1.61
Propoxy	1.43
Diethylaminoethoxy	0.80
Ethylsulphonyl	1.57

† Logarithm of equivalent reduction in infecting dose of virus.

but the derivatives were considered to be of approximately equal activity except for the diethylaminoethoxy derivative.

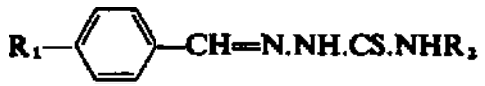
The parent compound, the 4-amino and the 4-methoxy derivatives were examined by a more precise method in which the compounds were tested at two dose levels against three doses of virus. The dose–response lines were calculated from the mean reciprocal survival times. At the lower dose level (0.006 mg) no difference in the activities could be detected, but at the higher dose level (0.06 mg) the parent compound was most active (1.76, compared with 0.92 for the 4-amino and 1.47 for the 4-methoxy derivatives).

The effect of the 4-amino derivative upon vaccinia virus *in vitro* was investigated. The compound was dissolved in 25% triethylene glycol in a concentration of 0.6 mg/ml and mixed with the virus; a control preparation was set up with virus in 25% triethylene glycol. The mixtures were held at room temperature for 2 hr, and the virus was sedimented and washed by centrifugation, and then inoculated into eggs. From the mean survival time of the embryos in the test and control groups it was evident that vaccinia virus was not inactivated by the compound *in vitro*.

In further experiments selected compounds were incorporated in the diet and administered to mice from 2 days before intranasal infection with vaccinia virus and for a period of 10–14 days afterwards. The results are

shown in Table 2. There were 6 survivors among 29 mice receiving the parent compound in an estimated daily dose of 10 mg/kg, and 15 among 30 receiving 5 mg/kg. The increase in proportion of survivors in the treated group was highly significant. Antiviral activity of the same order was observed with the 4-amino and 4-acetylamino derivatives. These derivatives and the parent compound were active at doses corresponding to one-half to one-quarter of the maximum tolerated dose. The 4'-*iso*butyl derivatives were less toxic than the parent compound, and good protection was obtained with 4-acetylaminobenzaldehyde 4'-*iso*butylthiosemicarbazone in one-quarter of the maximum tolerated dose.

TABLE 2. STRUCTURE–ACTIVITY RELATIONSHIPS OF 4- AND 4'-SUBSTITUTED BENZALDEHYDE THIOSEMICARBAZONES AGAINST VACCINIA VIRUS IN MICE. From Hamre *et al.* (1951)

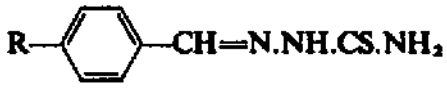


R_1	R_2	Daily intake (mg/kg)	Treated	Control	p‡
—	—	10	6/29†	0/30	<0.01
		5	15/30		<0.01
—	*Iso*butyl	650	3/10	1/20	0.05
		324	5/10		<0.05
Amino	—	22	8/30	1/50	<0.01
		10	7/30		<0.01
Amino	*Iso*butyl	210	4/10	1/20	<0.02
		109	3/9		>0.05
Acetylamino	—	43	17/40	2/70	<0.001
		22	5/39		<0.001
Acetylamino	*Iso*butyl	1447	7/8	0/10	<0.001
		713	19/20	2/30	<0.001
		170	3/10	2/20	<0.05
		54	2/9	2/20	>0.1
Methoxy	—	288	0/20	0/30	>0.05
		164	3/20		>0.05
Ethanesulphonyl	—	305	2/9	0/20	0.05
		185	1/10		<0.05
Butyl	—	166	1/9	0/20	<0.01§
		78	1/10		>0.05

† No. of survivors/No. in group.
‡ Probability of significance of increase in survivors in treated group.
§ Probability as stated by the authors.

From the results obtained in eggs and mice Hamre *et al.* selected benzaldehyde thiosemicarbazone, 4-acetylaminobenzaldehyde thiosemicarbazone and their 4′-*iso*butyl derivatives as being the most promising members of the series, but stressed the fact that the effective doses were fairly close to the maximum tolerated doses.

TABLE 3. EFFECT OF 4-SUBSTITUTED BENZALDEHYDE THIOSEMICARBAZONES AGAINST VACCINIA VIRUS IN MICE.
From Thompson *et al.* (1951)



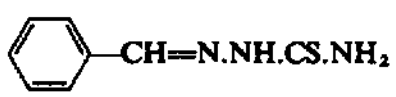
R	% in diet	Treated	Untreated
—	0.02	10/16	2/16
	0.03	8/18	4/18
	0.04	99/177	25/175
	0.08	10/15	2/16
Hydroxy	0.04	11/35	8/36
Amino	0.04	13/49	8/51
Acetylamino	0.04	5/18	1/18
	0.05	3/17	6/18
	0.06	0/18	4/18
	0.10	2/17	1/18
Dimethylamino	0.04	5/18	6/18
*Iso*propyl	0.04	1/18	3/18
Ethylsulphonyl	0.04	15/35	9/36
	0.06	3/18	3/18
*Iso*butoxy	0.025	5/18	3/18
	0.04	16/54	6/53

Thompson, Price and Minton (1951) investigated the antiviral effect of benzaldehyde thiosemicarbazone and some of its 4-substituted derivatives in mice infected with vaccinia virus by the intranasal route. The virus used was a neurotropic variant of the IHD strain and the compounds under test were incorporated in the diet. Treatment was begun at some time between 3 days before infection and 2 days afterwards, and was continued throughout the incubation period. The results are shown in Table 3. The parent compound was more active than any of the derivatives. No activity was found in similar tests with the 4-acetylamino, 4-dimethylamino and 4-ethylsulphonyl derivatives of benzaldehyde 4′-*iso*butylthiosemicarbazone.

It was therefore concluded that activity was reduced or abolished by substitution in the 4- and 4'-positions. The results confirm the observations of Hamre *et al.* in respect of the antiviral activity of benzaldehyde thiosemicarbazone in mice, but not of the relative activity of the derivatives. This discrepancy may be due to the difference in the route of infection used, and also in part to the fact that the test used by both groups of workers was insufficiently quantitative to enable the relative activities of the derivatives compared with the parent compound to be ascertained with sufficient precision. Thompson *et al.* (1951) were also able to confirm the observation of Hamre *et al.* that benzaldehyde thiosemicarbazone does not inactivate vaccinia virus on contact *in vitro*, and they therefore concluded that the compound acts by interfering with the multiplication of the virus, either by a direct action on the virus inside the cell, or by affecting the metabolic process of the host.

Thompson *et al.* (1953a) extended these observations to cover a much wider series of thiosemicarbazones. These will be described below in the section on other thiosemicarbazones, but this series also includes some other benzaldehyde thiosemicarbazones, and their findings with these compounds are shown in Table 4. The compounds were tested against 3 dose-levels of virus given by the intracerebral route. Further evidence was obtained of the activity of the parent compound, and the 4-nitro and

TABLE 4. EFFECT OF SUBSTITUTED BENZALDEHYDE THIOSEMICARBAZONES AGAINST VACCINIA VIRUS IN MICE. From Thompson *et al.* (1953b)



R	Position	% in diet	Treated			Control		
			2†	3	4	2	3	4
—	—	0.04	4/6‡	2/6	5/5	0/5	1/6	1/5
		0.08	5/5	2/5	3/5	0/5	1/6	1/5
Nitro	4	0.04	6/14	8/11	10/12	1/11	2/12	4/12
Nitro	3	0.04	0/6	3/3	1/6	0/6	0/6	1/6
Methoxy	2	0.06	9/12	9/12	11/12	0/12	0/12	7/12

† Negative logarithm of virus dose.
‡ No. of survivors/No. in group.

4-methoxy derivatives were equally active. No activity was found with benzaldehyde semicarbazone, a result which was in agreement with the earlier observations of Hamre *et al.* on the necessity of sulphur for antiviral activity. The following substituents also abolished the antiviral activity of benzaldehyde thiosemicarbazone; 4-phenyl, 3-nitro, 4-chloro, 3-chloro, 2-chloro, 2-hydroxy, 2-hydroxy-5-chloro, 3-methoxy-4-hydroxy-5-bromo. 3-Ethoxy-4-hydroxy-5-bromo-4-dimethylaminobenzaldehyde thiosemicarbazone methiodide was also inactive.

Benzaldehyde thiosemicarbazone was also thought to have some slight effect in rabbits infected intradermally with decimal dilutions of the IHD strain of vaccinia virus. The compound was given intraperitoneally in single daily doses of 100–150 mg for 6–8 days. The treatment did not prevent the formation of the local lesions, but generalization of the infection and necrosis at the site of the local lesions were prevented.

Thompson *et al.* (1953a) also demonstrated the antiviral activity of benzaldehyde thiosemicarbazone in tissue culture, but the methods available to them at the time were very crude by present day standards. A recent demonstration of activity by means of the plaque inhibition test is shown in Fig. 2.

Since 1953 very little further work has been done on the benzaldehyde thiosemicarbazones, since attention became concentrated on the isatin 3-thiosemicarbazones, which have higher activity and are better tolerated. The benzaldehyde thiosemicarbazones are nevertheless of great historical interest, as being the first true antiviral agents to be discovered, and have been described in detail for this reason; it is well within the bounds of possibility that they could have yielded an antiviral agent useful in human medicine if their development had not come to a halt.

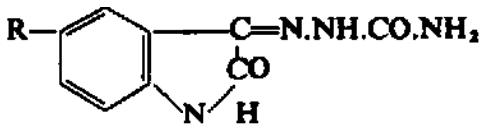
ISATIN 3-THIOSEMICARBAZONE AND DERIVATIVES

ACTION AGAINST VACCINIA VIRUS

In addition to benzaldehyde thiosemicarbazones, Thompson *et al.* (1953a) also investigated the antiviral activity of a wide range of thiosemicarbazones of aliphatic and aromatic ketones and aldehydes, which will be described in the section on miscellaneous thiosemicarbazones. These included a compound of great promise, isatin 3-thiosemicarbazone, the development of which has given rise to the antiviral compound methisazone.

The results obtained with isatin 3-thiosemicarbazone and its 5-methyl derivatives are shown in Table 5. The compounds were administered in the diet to mice infected intracerebrally with serial decimal dilutions of vaccinia virus. Almost complete protection against death was obtained with isatin 3-thiosemicarbazone given in the diet in concentrations of 0.08–0.16%. The 5-methyl derivative conferred no protection and isatin 3-semicarbazone was also inactive. These results show that isatin 3-thiosemicarbazone is at least as effective as benzaldehyde thiosemicarbazone. They also confirm the requirement for sulphur in the side-chain, and show in addition that antiviral activity is abolished by substitution of a methyl group in the 5-position.

TABLE 5. EFFECT OF ISATIN 3-THIOSEMICARBAZONE AND ITS 5-METHYL DERIVATIVES AGAINST VACCINIA VIRUS IN MICE.
From Thompson *et al.* (1953b)



R	% in diet	Treated			Control		
		2†	3	4	2	3	4
—	0.08	4/6‡	4/6	5/6	0/6	1/6	2/6
	0.12	6/6	4/6	5/5	0/6	2/6	1/6
	0.16	6/6	4/6	6/6	0/6	1/6	4/6
Methyl	0.10	0/6	0/6	0/6	1/5	2/6	1/6

† Negative logarithm of virus dose.
‡ No. of survivors/No. in group.

Thompson *et al.* (1953b) also determined the amount of virus present in the brains of mice infected with vaccinia virus and treated with isatin 3-thiosemicarbazone. The titre 5 days after infection was 5.1 log units, compared with 5.8 log units in untreated controls. In a similar experiment the titre was 2.4 log units in the treated animals and 3.4 log units in the controls. Although the reduction in titre of virus produced by treatment is small, it is sufficient to confer protection against death, and is unequivocal evidence of antiviral effect.

FIG. 2. Inhibition of plaque formation of rabbitpox virus in HeLa cells by benzaldehyde thiosemicarbazone.

reduction of 99.99% in the infecting dose of virus. The protection index was proportional to the dose of compound, and rose from 1 log unit with doses of 0.05 mg to the maximum of around 4 log units with doses of 0.5 mg. The protection index was not significantly increased by further increasing the dose.

In further work the effect of reducing the number of doses was investigated, and also the effect of varying the time of administration in relation to the time of infection, since it was observed that isatin 3-thiosemicarbazone was fairly insoluble and remained for some time at the site of injection, a fact which made it likely that an adequate blood concentration could be obtained with as few as 1 or 2 doses. The results are shown in Table 7. The mice were all infected intracerebrally with a dose of virus ranging from 100 to 1000 LD_{50}. Groups of 6 animals were treated with 1 or 2 doses of isatin 3-thiosemicarbazone ranging from 0.25 to 2.5 mg given subcutaneously, and 4 mice were left untreated as a control group. From a comparison of the mortalities it was seen that complete protection could not be obtained even with the maximum dose when given 3 hr after infection, whereas complete protection was obtained with 0.5 mg given 18 or 24 hr after infection. The incubation period with the dose of virus employed was 4–5 days. The compound therefore had a depôt effect of limited duration.

The immune state of treated animals which survived infection was investigated by giving an intracerebral challenge of 100 LD_{50} of vaccinia virus 18 days after the initial infection. The results are shown in Table 8.

TABLE 7. EFFECT OF LIMITATION OF PERIOD OF TREATMENT WITH ISATIN 3-THIOSEMICARBAZONE. From Bauer (1955)

Expt. No.	No. of doses	Time after infection (hours)	Dose (mg)						
			0	0.25	0.5	1.0	1.5	2.0	2.5
1	1	3	4/4†	6/6	2/6	2/6	3/6	5/6	2/6
2	1	3	4/4	5/6	3/6	3/6	3/6	4/6	2/6
3	2	1½, 18	4/4	2/6	0/6	1/6	0/6	0/6	0/6
4	2	18, 24	4/4	1/6	0/6	0/6	0/6	0/6	0/6
5	1	18	2/4‡	2/6	0/6	0/6	0/5	0/6	0/6

† No. of mice dying/No. in group.
‡ Morbidity 4/4.

Mice which survived infection as the result of treatment with isatin 3-thiosemicarbazone were solidly immune to reinfection by the intracerebral route. It was inferred from this that the virus was able to multiply sufficiently to induce an immune response. This is quite probable, but results obtained much later by Squires and McFadzean (1966) indicated that some local protective mechanism may have been induced. This will be discussed further in the section on the mechanism of action of the thiosemicarbazones.

TABLE 8. IMMUNE STATE OF MICE SURVIVING VACCINIA INFECTION AFTER TREATMENT WITH ISATIN 3-THIOSEMICARBAZONE. From Bauer (1955)

Initial infection			Challenge infection	
Virus dilution	Treated	Controls	Treated survivors	Challenge controls
10^4	6/6†	2/6	6/6	0/4
10^5	6/6	2/6	6/6	

† No surviving/No. in group.

The effect of treatment with isatin 3-thiosemicarbazone upon the multiplication of vaccinia virus was investigated. Mice were infected intracerebrally with 10–100 LD_{50} of vaccinia virus and assigned to two groups; one was treated with 2 mg of the compound subcutaneously twice daily for 4 days, and the other group was left untreated. At daily intervals 2 mice were selected from each group at random, and the content of virus in pooled suspensions of the brains was determined by intracerebral titration in mice, the titre being expressed as the LD_{50}. The results showed the rate of multiplication of the virus in the treated and control groups, and are shown in Table 9.

Treatment with isatin 3-thiosemicarbazone did not prevent the multiplication of the virus to titres which might exceed 5.5 log units, but the titres were in general 1 log unit lower than the titres in the control group. The reduction in titre produced by treatment, amounting to a 90% reduction in the amount of virus present, was sufficient to prevent the occurrence of symptoms of encephalitis, which do not appear until the titre exceeds 10^6.

TABLE 9. EFFECT OF TREATMENT WITH ISATIN 3-THIOSEMICARBAZONE UPON THE MULTIPLICATION OF VACCINIA VIRUS IN MICE INFECTED INTRACEREBRALLY. From Bauer (1955)

Infecting dose of virus (LD_{50})	Day	Titre (LD_{50})	
		Treated	Control
10^2	1	0.75	2.5
	2	3.75	3.75
	3	4.25	$\geqslant 5.5$
	4	$\geqslant 5.5$	6.25
10^3	1	?3.5	3.25
	2	$\geqslant 4.5$	$\geqslant 4.5$
	3	5.5	$\geqslant 6.5$
	4	5.25	$\geqslant 6.5$
10^4	1	1.5	3.25
	2	3.25	3.5
	3	4.75	6.25

In discussing the above results Bauer concluded that treatment with isatin 3-thiosemicarbazone during the incubation period of smallpox might confer immediate protection to persons who had been in contact with the infection, and that the compound might be of value as a prophylactic agent in epidemics of smallpox. This prophetic suggestion was not put to the test until 1963, when the derivative 1-methylisatin 3-thiosemicarbazone was shown to have a prophylactic effect against smallpox in a trial carried out in Madras (Bauer *et al.*, 1963).

The introduction of continuous cell lines made it possible to examine the effect of isatin 3-thiosemicarbazone against vaccinia virus in tissue culture by methods more precise than those available to the early workers in the field. Sheffield, Bauer and Stephenson (1960) used the ERK-1 line of HeLa cells. Monolayers were grown in medium containing a range of concentrations of isatin 3-thiosemicarbazone, and after incubation for 7 days were examined microscopically for evidence of toxicity. The cells withstood a concentration of 40 μM with only minimal visible changes, and this concentration was selected as the maximum tolerated concentration in subsequent chemotherapy tests. Groups of 5 tube cultures containing about 10^5 cells were prepared, and the medium was removed and replaced with 1 ml of medium containing isatin 3-thiosemicarbazone in concentrations of 80, 24, 2.4 and 0.8 μM. A similar series of tubes was prepared, and

the medium was replaced with 1 ml of medium not containing the compound. After incubation at 37°C for 30 min, 1 ml of a series of dilutions of vaccinia virus was added to the cultures of each group, thus bringing the final concentrations of the compound to the range 40–0.4 μM. A control series of cultures was also prepared; these were infected similarly, but were treated with medium not containing isatin 3-thiosemicarbazone. The cultures were incubated for 7 days and then examined for the occurrence of cytopathic effect due to virus infection. From the results the titres of virus in the absence of the compound and in the presence of varying concentrations could be calculated. Protection was observed in cultures incubated in the presence of isatin 3-thiosemicarbazone; it was generally proportional to the concentration of compound present; a reduction in titre of 4.7 log units was obtained with a concentration of 40 μM.

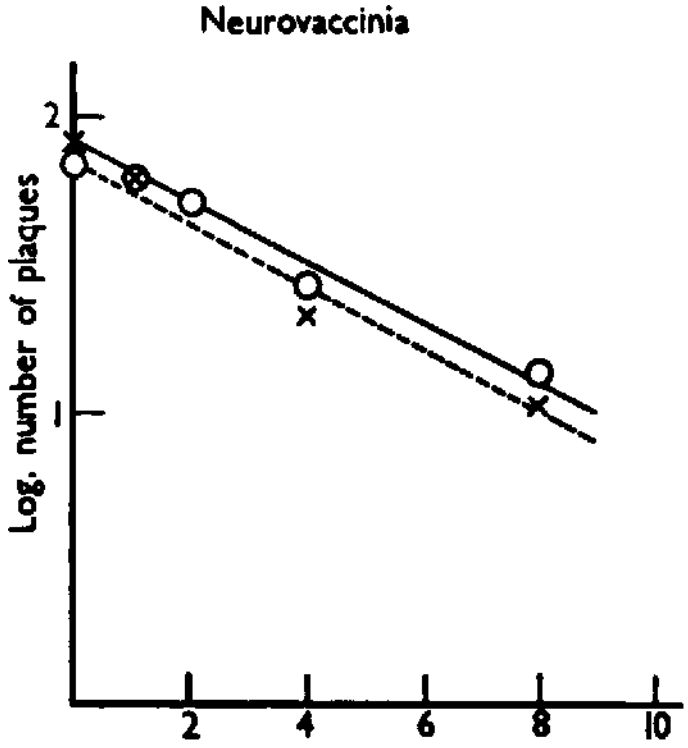


FIG. 3. Rate of inactivation of neurovaccinia virus in the presence of isatin 3-thiosemicarbazone. The virus was incubated at 37°C in medium containing the compound in a concentration of 40 μM (—○—) and in growth medium alone (— × —). Samples were removed at intervals and titrated by plaque counting. Inactivation proceeds at the same rate whether the compound is present or not, showing that the antiviral effect of isatin 3-thiosemicarbazone is not due to contact inactivation (Sheffield *et al.*, 1960).

Experiments were then carried out to determine whether the protection exerted by the compound was due to an action on the virus outside the cell. Suspensions of vaccinia virus containing 4×10^3 plaque-forming units were made in normal medium and also in medium containing isatin 3-thiosemicarbazone in a concentration of 40 μM. The preparations were incubated at 37°C; samples were taken at intervals up to 8 hr, and the titre of virus was determined by plaque counting. The results are shown in Fig. 3. The titre of the virus in logarithmic units fell linearly with time. The regression coefficients in the presence of isatin 3-thiosemicarbazone (−0.1048) and in its absence (−0.0989) differred significantly ($p < 0.001$) from zero, an observation which showed that thermal inactivation of virus was taking place, but they did not differ significantly ($p = 0.7$) from each other. The compound was therefore not inactivating the virus.

In further experiments the effect of isatin 3-thiosemicarbazone upon the uptake of vaccinia virus by ERK-1 (HeLa) cells was studied. A suspension of virus containing about 2×10^3 plaque-forming units and isatin 3-thiosemicarbazone (40 μM) was added in volumes of 0.5 ml to tube cultures of ERK-1 cells, which were then incubated at 37°C to allow adsorption of virus to occur. At intervals up to 24 hr, groups of cultures were removed and the virus suspension was washed off. Medium not containing the compound was placed on the cultures and incubation was continued for 40 hr. The cultures were then stained and the plaques which had developed were counted; the result indicated the number of plaque-forming units of virus which had adsorbed to the monolayer. A similar series of control cultures was set up in which adsorption of virus was allowed to take place in the absence of the compound. The results are shown in Fig. 4, from which it is evident that the presence of isatin 3-thiosemicarbazone in the medium did not impede the uptake of virus by the cells.

This work clearly shows that isatin 3-thiosemicarbazone does not inactivate extracellular virus and does not affect the uptake of virus by the cells. It was concluded that the site of action of the compound was intracellular. This will be discussed further in the section on mode of action, but all further work has confirmed this original conclusion.

Sheffield (1962) studied the toxicity of isatin 3-thiosemicarbazone for ERK-1 cells in greater detail. Cultures were grown in medium containing the compound in concentrations of 20, 40 and 60 μM. At intervals up to 5 days, sample cultures were removed from incubation, and determinations were made of the number of cells and the protein content of the cultures. The cell number and protein content were depressed by the compound in all the concentrations tested. After 5 days, cultures grown in the presence

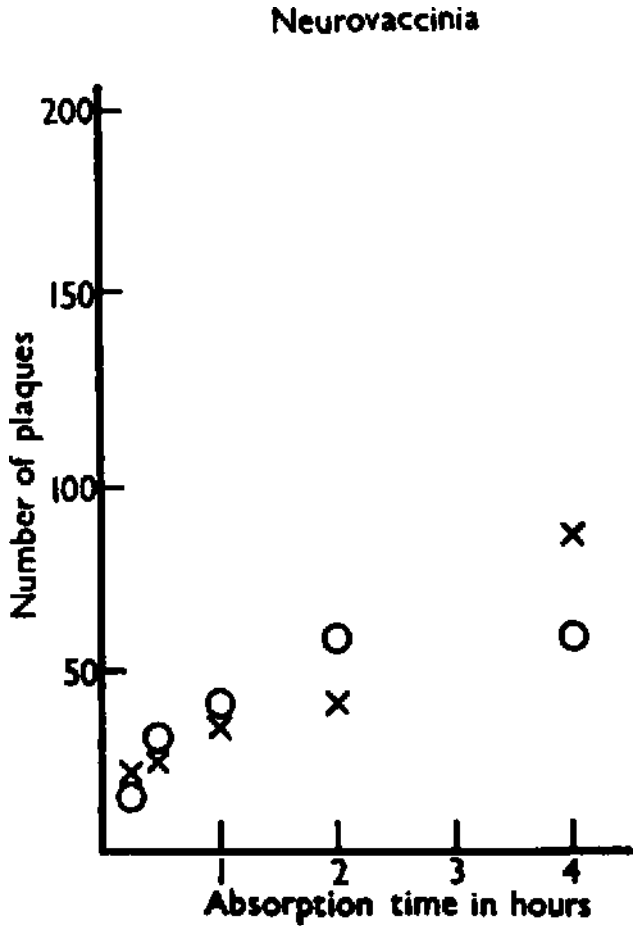


FIG. 4. Adsorption of neurovaccinia virus to HeLa cell monolayers in growth medium (—o—) and in growth medium containing 40 μM isatin 3-thiosemicarbazone (—x—). Samples were removed at intervals and titrated by plaque counting. The rate of adsorption is not affected by the compound, and the antiviral effect is therefore not due to blocking of uptake (Sheffield *et al.*, 1960).

of isatin 3-thiosemicarbazone in a concentration of 20 μM contained 20% less cells than the control cultures; this reduction in number was equivalent to an increase of 2 hr in the mean cell division time. It was concluded that the compound did not exert its antiviral effect as the result of an unspecific depressant effect upon cell metabolism, particularly since exposure to the compound for 24 hr, a period long enough to cover one cycle of virus multiplication, did not produce any detectable effect upon cell number or

protein content. In support of this conclusion it was found that poliomyelitis and Newcastle disease viruses would grow readily in the presence of concentrations of the compound as high as 40 μM. This observation is not supported by subsequent work of Lwoff and Lwoff (1964) who found that the multiplication of type 1 poliomyelitis virus was inhibited by a concentration of 40 μM.

The action of isatin 3-thiosemicarbazone on vaccinia virus growing in tissue culture was further investigated by Wright and Sagik (1964). Cultures of chick embryo kidney and HeLa cells were incubated with medium containing the compound in concentrations of 1, 2 or 5 μg/ml, and the content of DNA, RNA and protein were determined in sample cultures taken initially and after 24 and 48 hr. Concentrations of 1 μg/ml (4.5 μM) and 2 μg/ml (9 μM) had little effect in comparison with values obtained with control cultures not treated with the compound. Some depression was noted in all values in cultures exposed to 5 μg/ml (22.5 μM). The results for protein synthesis are in agreement with those reported by Sheffield (1962). Cultures which had been infected with vaccinia virus and incubated in the presence of the compound were sonically disrupted to release intracellular virus, and the yield of virus was determined by plaque counting on plates of chick embryo kidney cells. The results are shown in Table 10. The formation of infectious virus is reduced in both cell types in concentrations which had previously been shown to have little or no effect on cell metabolism. The reduction was generally proportional to the concentration of drug and the results afford an approximate dose–response curve.

The antiviral effect of isatin 3-thiosemicarbazone was demonstrated further in a different type of experiment. A suspension of HeLa cells

TABLE 10. EFFECT OF ISATIN 3-THIOSEMICARBAZONE ON THE MULTIPLICATION OF VACCINIA VIRUS IN TISSUE CULTURE. From Wright and Sagik (1964)

Concentration of drug		Virus yield (PFU/ml × 10^4) at 48 hr	
μg/ml	μM	Chick embryo kidney cells	HeLa cells
0	0	240	30
1	4.5	31	11
2	9.0	14	3.5
5	22.5	4	2.0

infected with vaccinia virus was prepared and diluted appropriately; 50 infected cells were placed on plates containing monolayers of chick embryo kidney cells, and an agar overlay was then put on which contained the compound in concentrations of 1, 2 and 5 μg/ml. The plates were then incubated in order to allow plaques to develop around the infected cells. The results are shown in Table 11. Isatin 3-thiosemicarbazone caused marked suppression of infective centre development at all the levels tested. The compound was dissolved in acetone before addition to the overlay medium, and a control was therefore included in which the overlay contained the equivalent amount of solvent.

TABLE 11. EFFECT OF ISATIN 3-THIOSEMICARBAZONE ON INFECTIVE CENTRE FORMATION IN CHICK EMBRYO KIDNEY MONOLAYERS SEEDED WITH 50 HELA CELLS INFECTED WITH VACCINIA VIRUS.
From Wright and Sagik (1964)

Overlay content	No. of plaques on plate				
Growth medium	15	21	14	12	14
Drug solvent	19	15	11	14	15
1 μg/ml drug	3	2	2	0	4
2 μg/ml drug	2	0	0	0	0
5 μg/ml drug	1	0	0	0	0

The effect of treatment with isatin 3-thiosemicarbazone both alone and combined with virus infection upon the viability of the cells was next investigated. Suspensions containing a known number of cells were plated and the number of colonies of cells (clones) which developed on incubation were counted. The results are shown in Table 12. When the cells were incubated in growth medium the number of clones obtained from 50 cells ranged from 11 to 26. This number was not reduced when acetone was added to the medium. The number was essentially unaffected when the cells were grown in medium containing 1 μg/ml isatin 3-thiosemicarbazone, but somewhat lower counts were obtained with concentrations of 2 and 5 μg/ml. When the experiments were repeated with a suspension of cells infected with vaccinia virus no clones were obtained. This result shows that cells infected with vaccinia virus die, even if the formation of infective virus is prevented by isatin 3-thiosemicarbazone.

TABLE 12. EFFECT OF ISATIN 3-THIOSEMICARBAZONE ON THE ABILITY OF NORMAL AND INFECTED HeLa CELLS TO FORM CLONES.
From Wright and Sagik (1964)

Incubation medium					
Uninfected	Clones formed (expected No. = 50)				
Growth medium	26	19	23	11	20
Drug solvent	17	16	19	29	21
1 μg/ml drug	15	16	20	?	?
2 μg/ml drug	9	11	6	?	?
5 μg/ml drug	13	12	9	8	?
Infected	Clones formed (expected No. = 250)				
Growth medium	0	0	0	0	?
Drug solvent	0	0	0	0	0
1 μg/ml drug	0	0	0	?	?
2 μg/ml drug	1	0	0	0	0
5 μg/ml drug	0	0	0	0	0

? Number of clones could not be ascertained

The work described so far shows that isatin 3-thiosemicarbazone is active in tissue culture, in other words activity observed in animal experiments is probably due to the compound itself, and not to a metabolite, since tissue cultures have a more limited range of metabolic activity than the intact animal. The results also show conclusively that isatin 3-thiosemicarbazone does not act outside the cell, and that infection irrevocably leads to the death of the cell, even if the production of infective virus is suppressed by the compound.

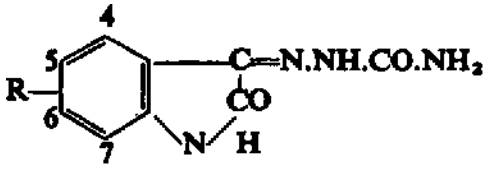
STRUCTURE–ACTIVITY RELATIONSHIPS OF ISATIN 3-THIOSEMICARBAZONE

In the early work on isatin 3-thiosemicarbazone, certain features of the structure–activity relationships of the molecule had already been observed. The necessity for sulphur in the side-chain was recognized by Thompson *et al.* (1953a), since isatin 3-semicarbazone was devoid of antiviral activity.

Substitution in the 5-position, as in 5-methylisatin 3-thiosemicarbazone, also abolished activity. Sheffield *et al.* (1960) confirmed the lack of activity of isatin 3-semicarbazone, and showed further that activity could also be abolished by replacing the sulphur atom with an imino (=NH) group (isatin 3-amidinohydrazone); 7-carboxyisatin 3-thiosemicarbazone sodium salt was also inactive.

The structure–activity relationships of isatin 3-thiosemicarbazone were subjected to a detailed study by Bauer and Sadler (1960a). Numerous derivatives substituted in the benzene ring, in the 1-position and in the side-chain were prepared, and their antiviral activity against vaccinia virus was assayed in comparison with that of the parent compound. The method used was a 4-point parallel line assay based on reciprocal survival time, and is described in detail in the section on antiviral test methods.

TABLE 13. EFFECT ON THE ANTIVIRAL ACTIVITY OF ISATIN 3-THIOSEMICARBAZONE OF INTRODUCING SUBSTITUENTS INTO THE AROMATIC RING.
From Bauer and Sadler (1960a)



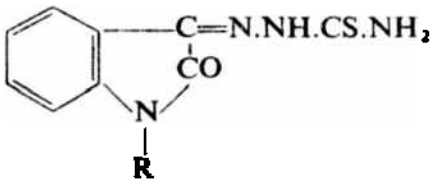
Substituent	Position			
	4	5	6	7
Amino	—†	0‡	—	—
Methyl	3.6	0	0.3	94
Ethyl	—	0	—	50
Carboxymethyl	—	0	—	—
Ethoxycarbonylmethyl	—	0	—	—
Hydroxy	—	0	—	—
Methoxy	—	0.03	0	—
Fluoro	—	35.5	43.1	20
Chloro	9.9	4.2	11.7	98.3
Bromo	67.3	3.1	10.5	16
Iodo	0	0	3.9	75.3
Carboxy	—	0	—	0
Trifluoromethyl	—	—	—	34.5
Nitro	—	0	—	0

† Not examined.
‡ Percentage activity (isatin 3-thiosemicarbazone = 100).

The compounds were first examined at a single dose level in mice infected with about 1000 LD_{50} of vaccinia virus. Compounds which protected against death or significantly prolonged the survival time in comparison with untreated controls were then examined more closely in a 4-point assay. The effect of introducing substituents into various positions of the aromatic ring is shown in Table 13, in which the antiviral activities of the compounds as determined in 4-point assays are expressed as percentages of the activity of the parent compound.

The results show that the substitutions in the aromatic ring usually result in reduction or total loss of antiviral activity. Substitution in the 5-position, except with fluorine, abolished activity, and substituents in the 4- and 6-positions also caused loss or reduction in activity in most cases. Activity was much less sensitive to substitution in the 7-position, the 7-methyl and 7-chloro derivatives having activities equal to that of the parent compound.

TABLE 14. EFFECT ON THE ANTIVIRAL ACTIVITY OF ISATIN 3-THIOSEMICARBAZONE OF INTRODUCING SUBSTITUENTS IN THE 1-POSITION.
From Bauer and Sadler (1960a)

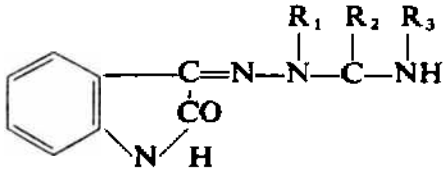


Substituent	Activity (Isatin 3-thiosemicarbazone = 100)
Methyl	202
Ethyl	286
*Iso*propyl	44
Propyl	28.5
Pentyl	3.4
Hydroxymethyl	42
1-Methyl-4-trifluoromethyl	48.4
2-Hydroxyethyl	204
Acetyl	87
Ethoxycarbonylmethyl	0
Diethoxycarbonylmethyl	0
2-Cyanoethyl	0
Carboxymethyl	0.02
5-Ethoxycarbonylmethyl-1-methyl	0
5-Carboxymethyl-1-methyl	0

In contrast, activity could be increased by suitable substitution in the 1-position of the pyrrolidine ring (Table 14). The activity of the 1-methyl derivative was twice, and of the 1-ethyl derivative nearly three times as high as that of the parent compound. The activity then fell off as the chain length of the alkyl substituent was further increased. These observations on the effect of alkylation in the 1-position upon antiviral activity led later to the practical development of 1-methylisatin 3-thiosemicarbazone (methisazone, Marboran) as a prophylactic agent against smallpox and as a drug for the treatment of vaccinia infections.

Investigation of the effect of substitution in the side-chain showed that modification in any position caused loss of antiviral activity (Table 15). It is evident that an intact side-chain is an absolute requirement.

TABLE 15. EFFECT ON THE ANTIVIRAL ACTIVITY OF ISATIN 3-THIOSEMICARBAZONE OF INTRODUCING SUBSTITUENTS IN THE SIDE-CHAIN.
From Bauer and Sadler (1960a)



R_1	R_2	R_3	Activity (Isatin 3-thiosemicarbazone = 100)
H	S	H	100
H	O	H	0
H	S	C_6H_5	0
H	NH	H	0
H	S	CH_3	0
C_6H_5	S	CH_3	?
C_6H_5	S	H	0
H	S	CH_2=CH.CH_2	4

The pyrrolidine ring of isatin 3-thiosemicarbazone was also essential for the retention of antiviral activity, since modifications in this region of the molecule all caused loss of activity. Thus, the carbonyl group in the 2-position was required, since 1-acetylindoxyl.thiosemicarbazone, in which the carbonyl group is reduced, was inactive. Extension of the side-chain in the 3-position by another carbon atom, as in 3-formyl-1-methyloxindole thiosemicarbazone, also caused loss of activity. The authors therefore

inferred that the active form of isatin 3-thiosemicarbazone was a tricyclic structure in which a third ring was formed by hydrogen bonding between the oxygen of the carbonyl group in the 2-position and the hydrogen in the 2'-position of the side-chain. However, it is uncertain whether such an intramolecular hydrogen bond would be stable in aqueous solution.

A number of miscellaneous compounds related to isatin 3-thiosemicarbazone were prepared and examined for antiviral activity, with negative results. They included isatin, thiosemicarbazide, isatin 3-amidinohydrazone (found inactive by Sheffield *et al.*, 1960), isatin 3-hydrazone, di-3-isatinazine, α-naphthisatin 3-thiosemicarbazone, β-naphthisatin 3-thiosemicarbazone and isatin 2-thiosemicarbazone. The lack of activity of isatin 2-thiosemicarbazone was of particular interest.

The results of the structure–activity investigation thus showed that it was possible to achieve an appreciable increase in the antiviral activity of the molecule by alkylation in the 1-position, and also confirmed in detail the isolated observations of previous workers that activity was reduced or abolished by modifications in the aromatic ring and the side-chain.

The activity of 1-ethylisatin 3-thiosemicarbazone was observed independently by Slack, Wooldridge, McFadzean and Squires (1964). The compound has been studied in further detail by Pollikoff (1965).

ACTIVITY OF ISATIN 3-THIOSEMICARBAZONE AND ITS DERIVATIVES ON VIRUSES OTHER THAN VACCINIA

In addition to vaccinia, the action of isatin 3-thiosemicarbazone and its derivatives has been investigated on other viruses belonging to the pox group, herpes, varicella-zoster, picornaviruses, adenoviruses and arboviruses.

POX VIRUSES

A classification of the pox viruses is shown in Table 16, which is taken from Andrewes (1964). Group I consists of closely related viruses with affinity to variola. Group III may be heterogeneous, containing viruses more properly belonging to groups I and II. Group VI contains viruses which have not yet been sufficiently characterized to be assigned to one of the foregoing groups.

The viruses which are known to be susceptible to chemotherapy with isatin 3-thiosemicarbazone and its derivatives all belong to group I.

TABLE 16. CLASSIFICATION OF THE POX VIRUSES. From Andrewes (1964).

Group I	Group II	Group III	Group IV	Group V	Group VI
Variola	Contagious pustular dermatitis	Sheeppox	Fowlpox	Rabbit myxoma	Molluscum contagiosum
Alastrim		Goatpox	Canarypox	Rabbit fibroma	Paravaccinia
Vaccinia	Bovine papular dermatitis	Lumpy skin disease		Squirrel fibroma	Yaba virus
Rabbitpox		Swinepox			
Monkeypox		Horsepox			
Ectromelia		Camelpox			
Cowpox					

Viruses belonging to the other groups have not so far been sufficiently investigated.

The first virus other than vaccinia to be investigated was ectromelia. Bock (1957) infected mice intranasally with ectromelia virus in a dose varying over a 100-fold range and treated them subcutaneously or intraperitoneally with isatin 3-thiosemicarbazone in a dose ranging from 25 to 250 mg/kg daily. No reduction in mortality or prolongation of survival time could be noted in comparison with untreated controls. Equally negative results were obtained when the virus was injected intracerebrally or into the footpad, and when the compound was given twice daily. A similar lack of activity in mice infected intracerebrally was reported by Bauer and Sadler (1960a).

In tissue culture, however, Sheffield *et al.* (1960) were able to demonstrate activity against ectromelia without difficulty. In an experiment of the type which they used for demonstrating activity against vaccinia it was found that isatin 3-thiosemicarbazone in a concentration of 40 μM would reduce the titre of ectromelia in the ERK-1 strain of HeLa cells by $10^{2.8}$. The appearances of protected and control cultures are illustrated in Fig. 5. Activity against ectromelia can also be conveniently demonstrated by incorporating the compound in an overlay and applying it to an infected monolayer. In the experiment illustrated in Fig. 6, a monolayer of HeLa cells was infected with ectromelia virus and covered with an overlay containing isatin 3-thiosemicarbazone in a concentration of 5 μM. A control preparation was set up with a plain overlay, and the cultures were incubated to allow plaques to develop. In the presence of the compound the formation of plaques was almost entirely suppressed. Isatin 3-thiosemicarbazone is clearly active against ectromelia, but the activity is not high enough to be detectable in experimental animals.

Certain dialkylthiosemicarbazones of isatin have a much greater activity against ectromelia, which can be easily demonstrated in animals. These compounds will be reviewed further on.

RABBITPOX

A considerable amount of chemotherapeutic work has been carried out with the Utrecht strain of rabbitpox, which was originally isolated from laboratory rabbits by Jansen (1941). At the time when isatin 3-thiosemicarbazone was still considered to be inactive against ectromelia it was naturally of considerable interest to survey the extent of its activity amongst other viruses of the pox group. The work of Bauer and Sheffield (1959)

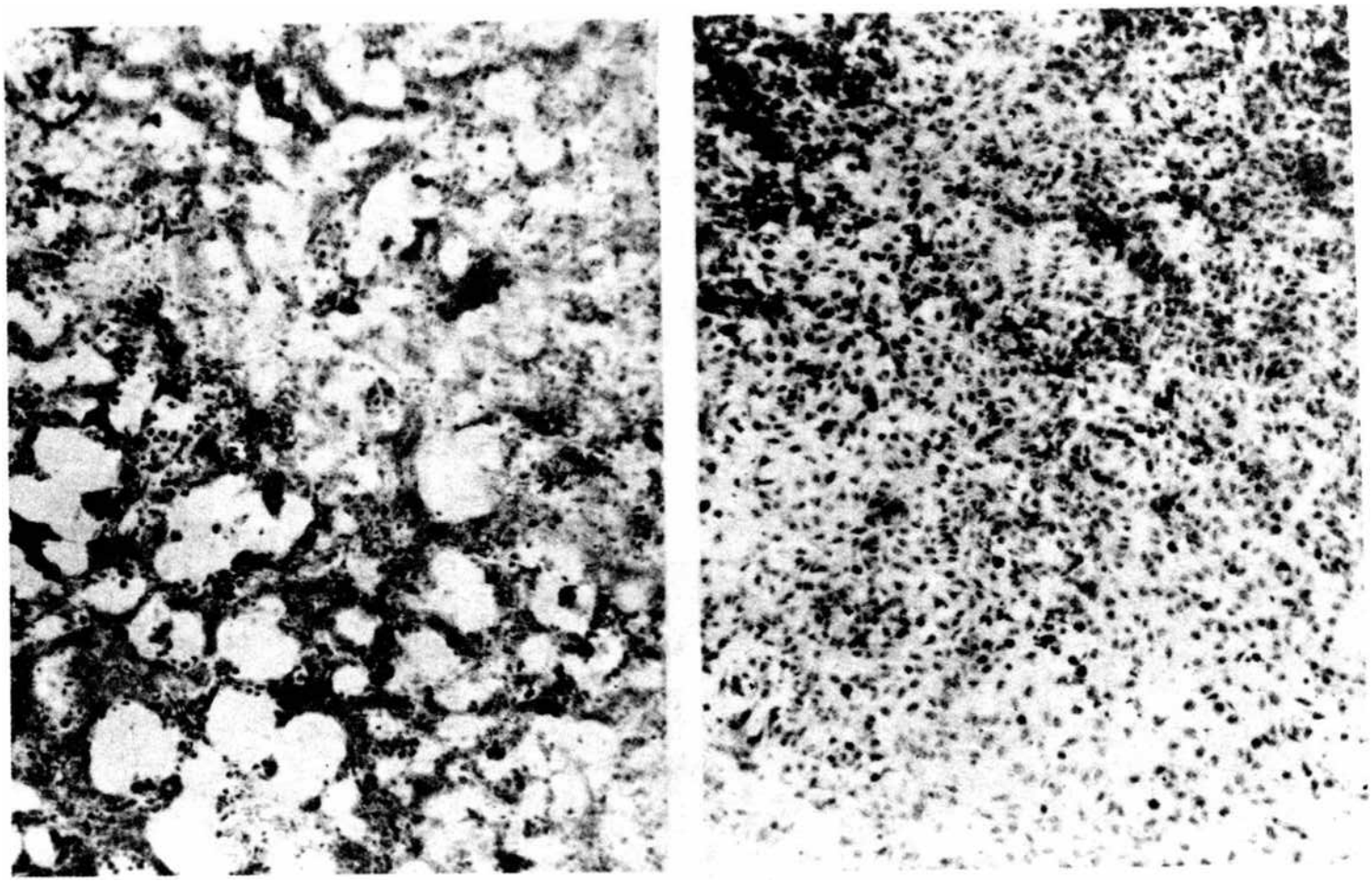


FIG. 5. Activity of isatin 3-thiosemicarbazone against ectromelia virus. *a*, cytopathic effect in a HeLa cell monolayer 3 days after infection; *b*, absence of cytopathic effect 3 days after infection in a monolayer exposed to isatin 3-thiosemicarbazone in a concentration of 40 μM.

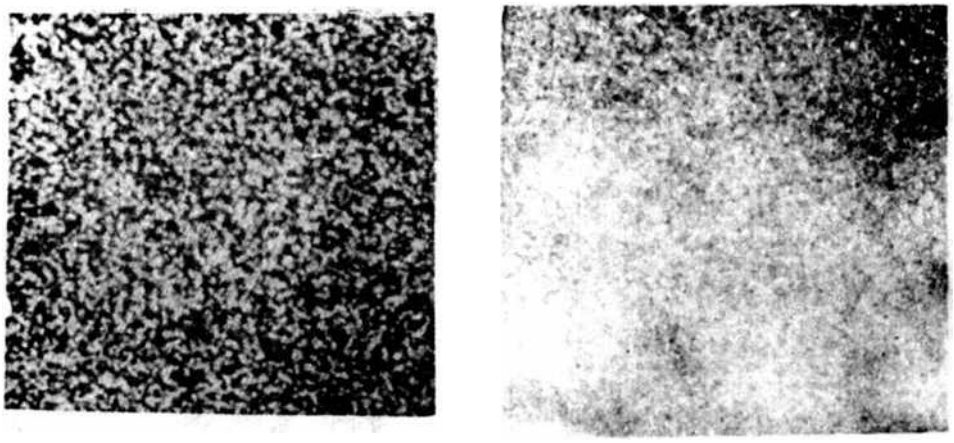


FIG. 6. Activity of isatin 3-thiosemicarbazone against ectromelia virus. *a*, plaque formation in a HeLa cell monolayer after incubation for 3 days under an agar overlay; *b*, absence of plaques in a monolayer under an agar overlay containing isatin 3-thiosemicarbazone in a concentration of 5 μM.

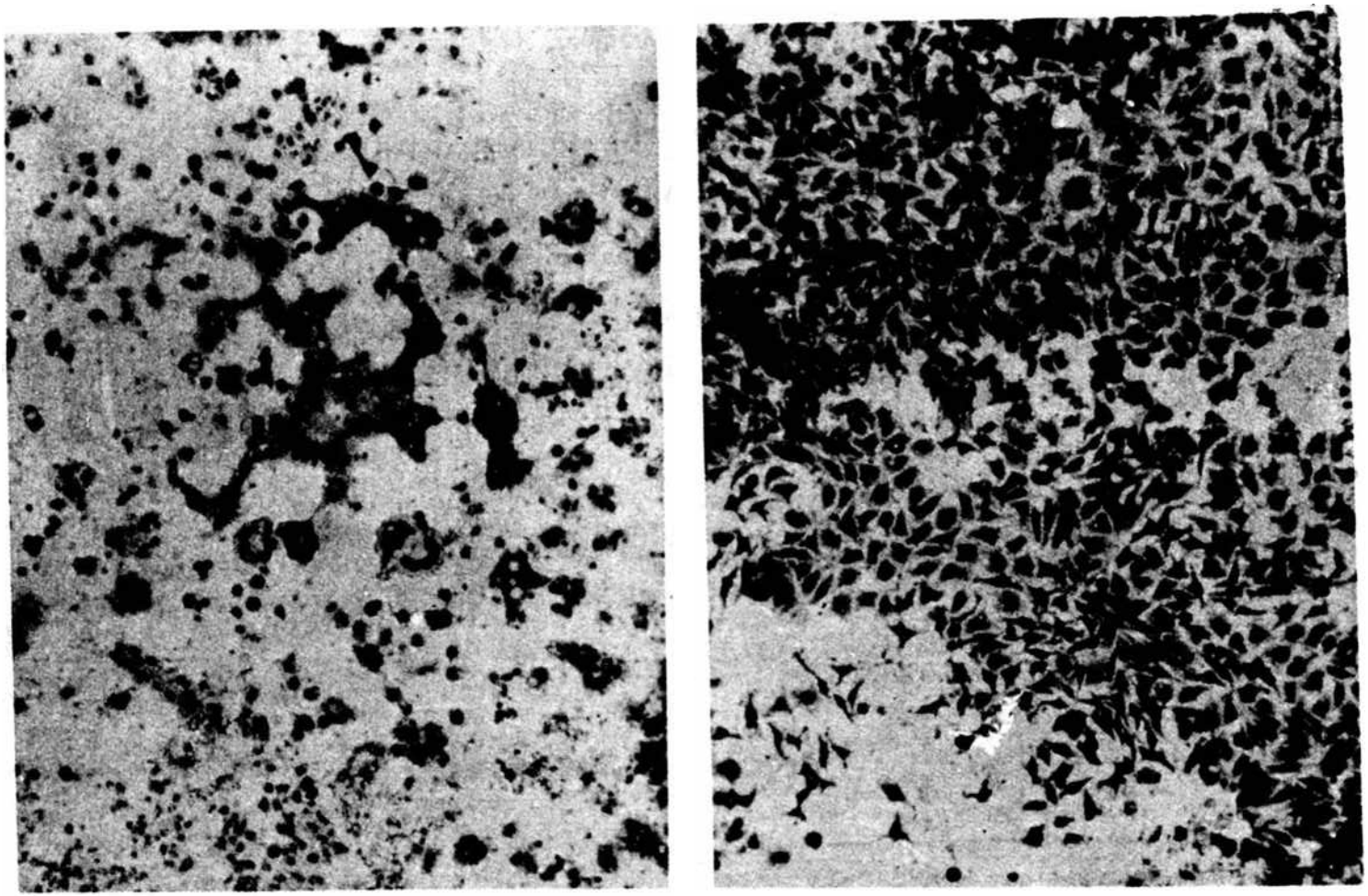


FIG. 7. Activity of isatin 3-thiosemicarbazone against rabbitpox virus. *a*, cytopathic effect in a HeLa cell monolayer 3 days after infection; *b*, absence of cytopathic effect 3 days after infection in a monolayer exposed to isatin 3-thiosemicarbazone in a concentration of 40 μM.

showed that it was highly active against rabbitpox. The virus regularly caused death on intracerebral injection in mice weighing less than 15 g. A suspension of mouse brain was titrated in parallel in decimal dilutions in 2 groups of 30 mice; one group was treated with isatin 3-thiosemicarbazone given subcutaneously twice daily in doses of 2 mg, and the other group was left untreated as a control. The treated mice all survived, whereas 27 of the control mice died of the virus infection. Consideration of the titres in the two groups showed that the compound had conferred protection against 100,000 LD_{50} of virus. The animals which survived the infection as the result of treatment received an intracerebral challenge injection of 1000 LD_{50} of rabbitpox virus 13 days later. All survived, and had therefore developed a high degree of immunity. The animals also survived a subsequent intracerebral challenge with vaccinia virus. The authors did not comment on the nature of the immunity so acquired, but it is by no means certain to be due to humoral immunity, since Squires and McFadzean (1966) showed that an interfering substance appeared in the brains of mice infected with vaccinia and protected from death by treatment with 3-methyl-4-bromo-5-formyl*iso*thiazole thiosemicarbazone (M and B 7714).

It was concluded by Bauer and Sheffield that pox viruses fell into two groups in respect of their sensitivity to isatin 3-thiosemicarbazone; vaccinia and rabbitpox were highly sensitive, whereas ectromelia was resistant. Subsequent work was to show that a gradation in sensitivity could be observed among the different pox viruses.

The sensitivity of rabbitpox virus to isatin 3-thiosemicarbazone was studied by Sheffield *et al.* (1960) in tissue culture in experiments of the type already described for vaccinia virus. Protection to the extent of $10^{4.6}$ TCD_{50} was observed in cultures of the ERK-1 strain of HeLa cells infected with serial dilutions of the virus. The protection against the cytopathic effect produced by the compound is illustrated in Fig. 7. The compound did not inactivate rabbitpox virus *in vitro*, and did not affect its adsorption on to HeLa cells; rabbitpox virus thus behaved similarly to vaccinia virus in all respects.

The effect of isatin 3-thiosemicarbazone on the growth cycle of rabbitpox virus in the ERK-1 line of HeLa cells was studied by Sheffield (1962). Replicate cultures were exposed for 1 hr to normal medium or medium containing the compound in a concentration of 20 μM. The medium was then removed and enough rabbitpox virus to infect all the cells was placed on the cultures. Adsorption of virus was allowed to continue for 4 hr at room temperature and the virus was removed and replaced with

normal medium or medium containing the drug in a concentration of 20 μM. Incubation was continued, and sample cultures were removed at intervals up to 72 hr. The supernatant fluids were removed and the cells were mechanically disrupted in order to release cell-associated virus. The amount of virus in the cells and culture fluids was then titrated by plaque counting. The results are shown in Fig. 8. In the control cultures the amount of virus present in the cells began to rise after 6 hr and attained a maximum titre of over 10^6 pfu/ml after 12–18 hr. In the cultures treated with isatin 3-thiosemicarbazone the growth of virus was completely suppressed. The titres of virus in the culture fluids behaved similarly at lower levels. Similar experiments (Fig. 9) were carried out with lower concentrations of the compound. Some virus was produced in the cells

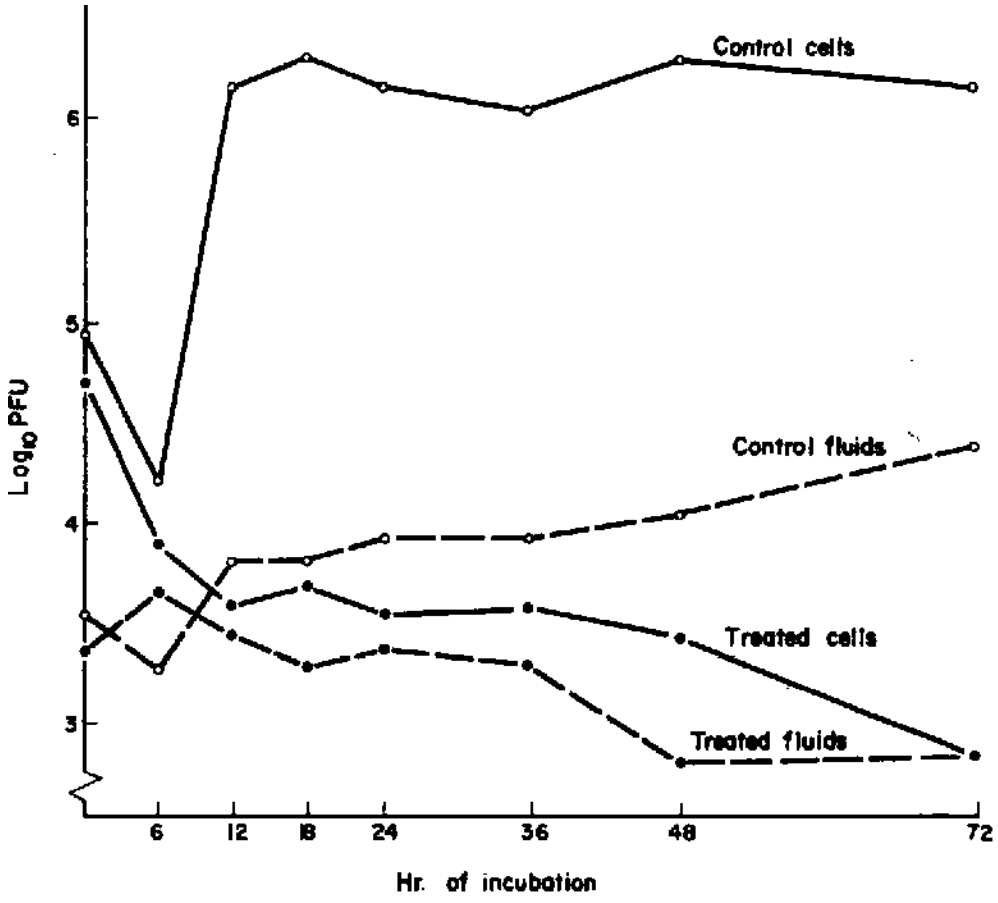


FIG. 8. Growth of rabbitpox virus in HeLa cell cultures incubated in normal growth medium (—○—) and in medium containing isatin 3-thiosemicarbazone in a concentration of 20 μM. The amounts of virus in the cells and supernatant fluid were determined separately by plaque titration of cultures taken at intervals (Sheffield, 1962).

in the presence of concentrations of 5 and 10 μM, but the titres were much below those of the control cultures.

Further work on the action of isatin 3-thiosemicarbazone on the multiplication of rabbitpox virus will be reviewed in the section on the mode of action of the compound.

A quantitative determination of the sensitivity of rabbitpox virus to isatin 3-thiosemicarbazone was made by Bauer (1961), who also examined the sensitivity of cowpox. A dose–response line for the compound was obtained by the method of Bauer and Sadler (1960a) in mice infected intracerebrally with rabbitpox virus (Fig. 10). The range of doses covered

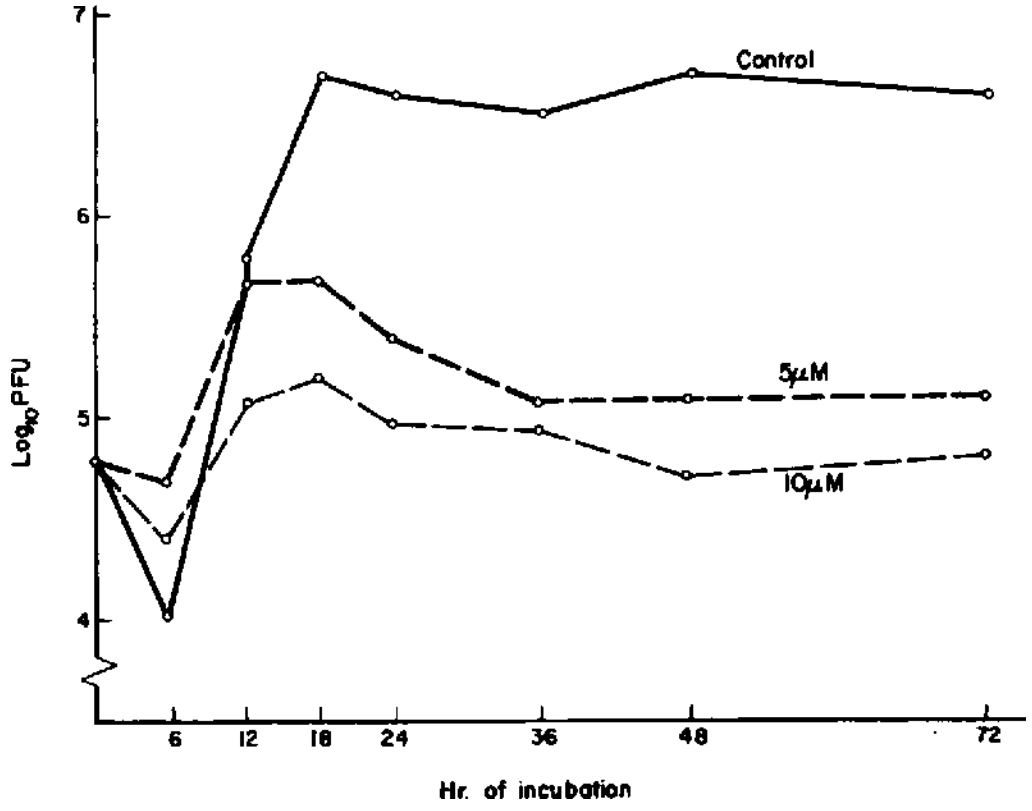


FIG. 9. An experiment of the type shown in Fig. 8. Inhibition of growth of rabbitpox virus in cultures of HeLa cells by isatin 3-thiosemicarbazone in concentrations of 5 μM and 10 μM (Sheffield, 1962).

by the line was 0.005–0.08 mg, whereas the range covered by the dose–response line with vaccinia virus was 0.02–0.16 mg. The doses required for protection against rabbitpox are therefore smaller than those required for protection against vaccinia, and the former virus is more sensitive. The properties of the dose–response line were examined further, and it

was found that it intersected the ordinate for the control group at a dose which was independent of the dose of virus used for infection. This dose at which no detectable protective effect could be observed was defined as the E_0 (zero effective dose), and it provided an absolute measure of the antiviral activity of a compound, thus obviating comparison with a standard and enabling the activity of different viruses to be compared against the same compound. The constancy of the E_0 is illustrated in Figs. 11 and 12, from which the mean values can be calculated. The E_0 for rabbitpox was 0.41 ± 0.17 mg/kg, compared with 0.75 ± 0.18 mg/kg for vaccinia. Rabbitpox was therefore nearly twice as sensitive as vaccinia.

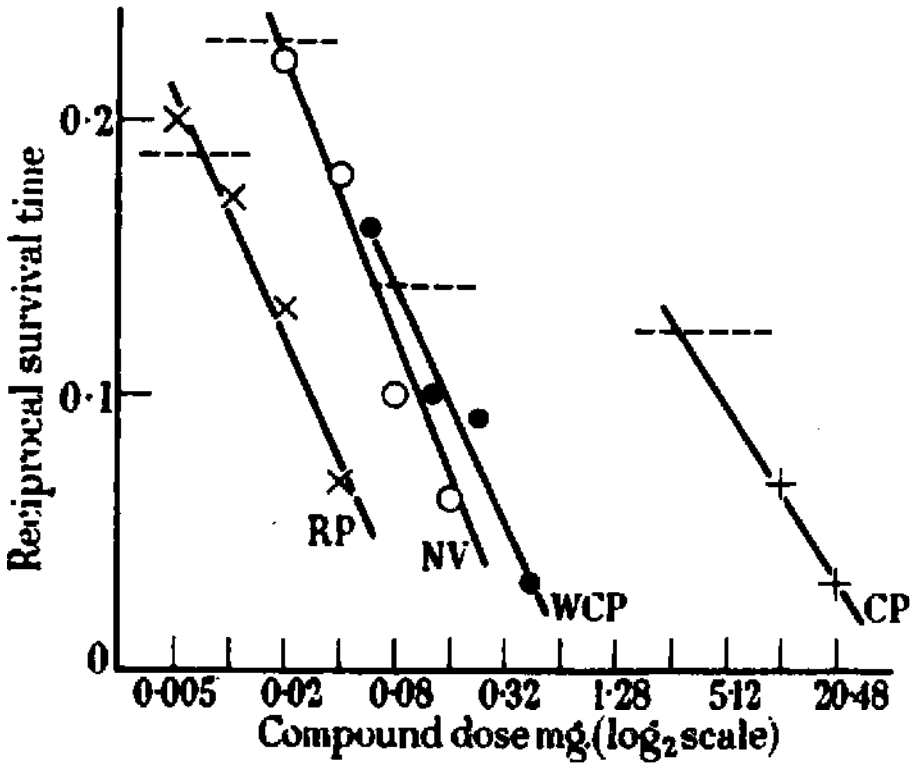


FIG. 10. Dose–response lines of the antiviral action of isatin 3-thiosemicarbazone against 4 pox viruses. Mice were infected intracerebrally and treated with the compound in the doses indicated. *Ordinate*: mean reciprocal survival time; *abscissa*: dose of compound on $\log_2$ scale. RP, rabbitpox; NV, neurovaccinia; WCP, white cowpox; CP, cowpox. The dotted lines indicate the mean value of the ordinates obtained in the absence of treatment (Bauer, 1961).

The strain of vaccinia virus used was a neurotropic variant of the IHD strain, but identical E_0 values were obtained with the WR and Williamsport strains of vaccinia virus.

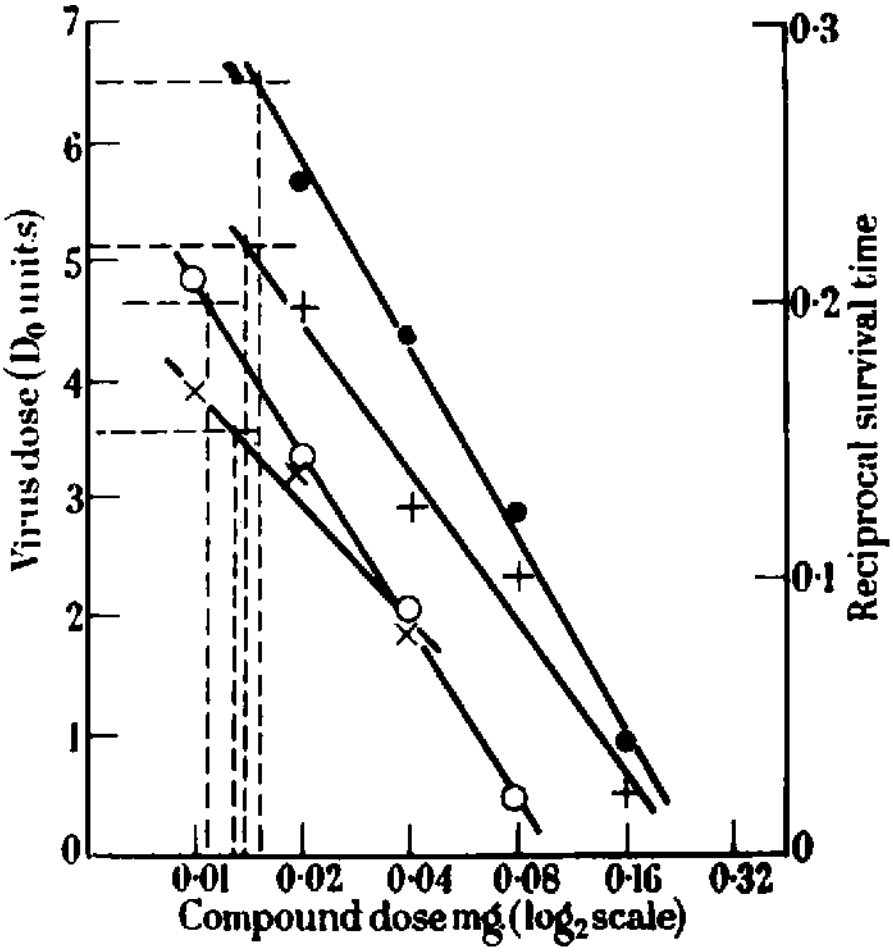


FIG. 11. Dose–response lines of the antiviral action of isatin 3-thiosemicarbazone against varying doses of neurovaccinia. Mice were infected intracerebrally with decimal dilutions of the virus and treated with the compound in the doses indicated. *Ordinates*: left, $\log_{10}$ dose of virus in D_0 units, right, mean reciprocal survival time; *abscissa*: dose of compound on $\log_2$ scale. The horizontal dotted lines indicate the mean value of the ordinates obtained in the absence of treatment. The point of intersection with the dose–response lines occurs at a dose (E_0) which is essentially constant whatever the dose of virus (Bauer, 1961).

COWPOX

Cowpox virus exists in two variants, red and white cowpox, which are so named according to the colour of the pocks which it produces on the

chorioallantoic membrane. Both variants can be adapted to intracerebral passage in mice, and dose–response lines and E_0 values for them are included in Figs. 10 and 12. The E_0 for white cowpox was 3.34 ± 0.54 mg/kg and for red cowpox 159.7 ± 54.5 mg/kg. White cowpox is thus less sensitive than vaccinia, and red cowpox very much less sensitive. The pox viruses so far investigated can therefore be arranged in order of sensitivity, as follows: ectromelia $\ll$ red cowpox $<$ white cowpox $<$ vaccinia (three strains) $<$ rabbitpox. Chemotherapeutic sensitivity is thus a continuously

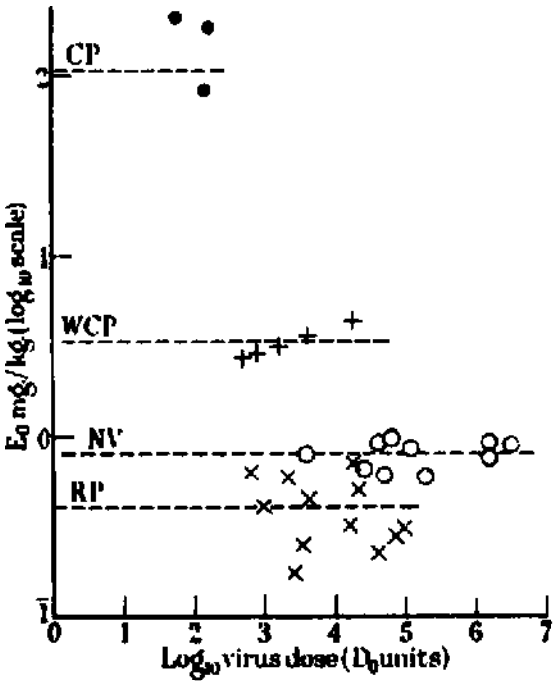


FIG. 12. Values of E_0 of isatin 3-thiosemicarbazone for 4 different pox viruses. *Ordinate*: E_0 in mg/kg on $\log_{10}$ scale; *abscissa*: $\log_{10}$ dose of virus used for infection in D_0 units. RP, rabbitpox; NV, neurovaccinia; WCP, white cowpox; CP, cowpox. The dotted lines indicate the respective mean values of E_0.

variable quantity. The most reasonable explanation of this observation was considered to be that isatin 3-thiosemicarbazone was not exerting its antiviral action by interfering with a function of the host cell, since the same host cells were involved in each infection, but against some component of the virus, which might well differ in detail at the molecular level between one virus and another, thus affecting the goodness of fit of the compound against the sensitive region of the virus material.

SMALLPOX

Smallpox virus exists in three variants, variola major, variola minor or alastrim, and a variant found in East Africa. Variola major is a severe disease with an average mortality of 30%, and occurs in endemic form in Africa and the East. Alastrim is a mild disease with a mortality not higher than 0.5%, which is endemic in South America. Variola major and alastrim viruses are very similar in their laboratory properties, but can be distinguished by special tests. They can both be adapted to intracerebral passage in infant mice, and have been extensively studied in chemotherapy experiments.

ALASTRIM

From work on the chemotherapy of vaccinia infection with isatin 3-thiosemicarbazone it seemed probable that this compound would be effective in the prophylaxis of smallpox, and an essential preliminary step was the investigation of its activity against smallpox virus in animal experiments. It was by no means certain that the compound would be effective, in view of its relative lack of activity against ectromelia and reduced activity against cowpox. It was therefore necessary to determine the position of the two variants of smallpox virus in the spectrum of sensitivity of the pox viruses.

Bauer and Sadler (1960b) selected 1-ethylisatin 3-thiosemicarbazone, the derivative which had the highest activity against vaccinia, and investigated its action against the Schofield strain of alastrim virus in infant mice. In mice up to 6 days of age intracerebral injection of the virus produces a fatal encephalitis; in older mice signs of encephalitis develop but many animals recover, and mice weighing 10 g or more show no signs of illness.

The work was carried out with litters of mice of the same age and not more than 6 days old. The litters were pooled and redistributed to the mothers, and the mice were then infected intracerebrally with alastrim virus in a dose of 100 mouse LD_{50} or 10^4 pock-forming units contained in a volume of 0.01 ml. The mice were weighed, and the dose of 1-ethylisatin 3-thiosemicarbazone selected for each group was injected subcutaneously in a volume which usually lay within the range of 0.01–0.03 ml. Infection and dosing were carried out with a micrometer syringe. A control group of mice was infected similarly and left without treatment. The results are shown in Table 17. The control mice developed cerebral irritation after 4–5 days and died after 5–10 days; 60 of 80 animals died,

and 12 of the remainder developed encephalitis and recovered. Among 118 animals treated with 1-ethylisatin 3-thiosemicarbazone in doses ranging from 0.25 to 250 mg/kg there were 10 deaths, all in mice receiving

TABLE 17. EFFECT OF 1-ETHYLISATIN 3-THIOSEMICARBAZONE AGAINST ALASTRIM INFECTION IN INFANT MICE. From Bauer and Sadler (1960b)

Dose level (mg/kg)	Treated			Control			
	No.	Died	Remained well	No.	Died	Developed encephalitis and recovered	Remained well
250	9	0	9	8	7	0	1
25	9	0	9	9	9	0	0
10	16	0	16	14	11	3	0
5	26	4	22	27	20	0	7
2.5	19	1	18	20	11	5	4
1.25	11	1	10	12	8	0	4
1.00	6	0	6	6	5	1	0
0.50	14	1	13	14	10	4	0
0.25	8	3	5	8	5	3	0

doses of 5 mg/kg or less; the remaining animals showed no signs of illness. The dose level which conferred 50% protection against infection was around 0.25 mg/kg. The compound was thus effective in protecting mice against intracerebral infection with alastrim virus, and the authors concluded that it might be of value in the treatment of smallpox in man.

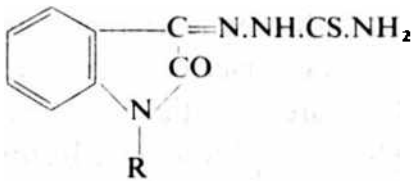
In similar unpublished experiments protection was also obtained with isatin 3-thiosemicarbazone (5 mg/kg) and its 1-methyl derivative (5 and 10 mg/kg). Mice which survived infection as a result of treatment were solidly immune to subsequent challenge infection.

VARIOLA MAJOR

From the observation that alastrim infection was sensitive to isatin 3-thiosemicarbazone and certain of its derivatives it does not necessarily follow that these compounds are equally active, or active at all, against variola major, and this point was therefore put to the test by Bauer *et al.* (1962). The work was carried out with the Harvey strain of variola major

virus, the international reference strain. In preliminary experiments it was found that this virus would produce a reproducible fatal encephalitis in infant mice 2–6 days of age. The mice were infected intracerebrally with 10^5–10^6 pock-forming units of virus, a dose which corresponded to 10^3–10^4 mouse LD_{50}. They were then dosed subcutaneously in the manner described previously in the experiments with alastrim. The results are shown in Table 18. Isatin 3-thiosemicarbazone conferred complete protection in a dose of 25 mg/kg; the few deaths which occurred at higher dose levels were probably due to toxicity. The dose conferring 50% protection lay between 5 and 10 mg/kg. Complete protection was obtained with the 1-methyl derivative in a dose of 25 mg/kg, and almost complete protection with doses down to 5 mg/kg; the dose conferring 50% protection lay between 2.5 and 5 mg/kg. No signs of toxicity were observed with doses

TABLE 18. EFFECT OF ISATIN 3-THIOSEMICARBAZONE AND 1-SUBSTITUTED DERIVATIVES AGAINST VARIOLA MAJOR INFECTION IN INFANT MICE. From Bauer *et al.* (1962)



R	Dose (mg/kg)	Treated	Control
H	100	2/5†	5/5
	50	1/10	13/13
	25	0/4	5/5
	10	1/5	6/6
	5	4/6	
Methyl	50	0/4	5/5
	25	0/3	5/5
	10	1/11	12/12
	5	1/12	12/12
	2.5	8/14	16/16
Ethyl	100	0/5	5/5
	50	2/20	20/20
	25	2/10	11/11
	20	3/11	22/22
	10	3/6	6/6
	5	5/15	14/14

† No. dying/No. in group.

as high as 50 mg/kg. The 1-ethyl derivative had a marked protective effect down to 20 mg/kg, but the results were somewhat irregular. The compounds were also studied in lower doses. Animals treated with isatin 3-thiosemicarbazone in doses of 2.5 mg/kg failed to survive the infection, but the survival time was prolonged in comparison with untreated controls. A dose of 1.5 mg/kg was ineffective. Three of 6 animals treated with the 1-methyl derivative in a dose of 2.5 mg/kg survived, and the survival time of the remainder was prolonged. A similar result was obtained in animals treated with the 1-ethyl derivative in doses of 5 and 10 mg/kg.

Animals which had survived infection as a result of treatment with an active compound were challenged intracerebrally 16 days later with 10^5 pock-forming units of rabbitpox virus. This virus was selected since the animals were at that time too old to be susceptible to variola virus. All the animals survived. During treatment sufficient multiplication of virus had taken place to stimulate the development of immunity, as had been found earlier (Bauer, 1955) with vaccinia virus.

The authors also made some observations on the structure–activity relationships of isatin 3-thiosemicarbazone against variola major virus. Isatin 3-semicarbazone was inactive in a dose of 100 mg/kg; sulphur in the side-chain was therefore essential for activity. No activity was found with 5-carboxymethylisatin 3-thiosemicarbazone, and activity was therefore abolished by substitution in the 5-position. Substitution in the 7-position was consistent with retention of activity, since 7-methylisatin 3-thiosemicarbazone conferred complete protection in a dose of 10 mg/kg, and some effect was obtained with 1-chloroisatin 3-thiosemicarbazone in doses of 2.5 and 10 mg/kg. Protection was observed with 1-β-hydroxyethylisatin 3-thiosemicarbazone in doses of 5 and 10 mg/kg, but the activity was not so high as that found against vaccinia. The structure–activity relationships of isatin 3-thiosemicarbazone against variola major virus were thus generally similar to those established with vaccinia. Variola major is related to vaccinia and rabbitpox in its chemotherapeutic sensitivity, and not to cowpox and ectromelia. It is less sensitive than alastrim.

METHISAZONE

The work described in the foregoing section led to the development of several derivatives of isatin 3-thiosemicarbazone which had higher antiviral activity than the parent compound. It also showed that variola major and alastrim viruses possessed adequate sensitivity to these chemotherapeutic agents, and the way was therefore clear for a trial of one of these com-

pounds in man. The compound selected was 1-methylisatin 3-thiosemicarbazone (Fig. 13), known later by the approved name of methisazone and the brand name of 'Marboran'. Although it is not the most active member of the series, practical factors such as ease and cost of preparation gave it some advantage over related compounds.

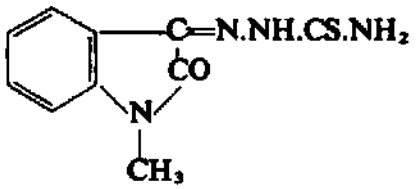


FIG. 13. Structural formula of methisazone.

Before a compound becomes a drug and is ready for trial in man, thorough studies must be made of its toxicity in animals and of its uptake, distribution, metabolism and excretion. These aspects will not be considered until later. It is also necessary to produce a formulation of the drug which is a satisfactory and acceptable pharmaceutical preparation. In the early work with methisazone in man the drug was used in the form of compressed tablets, but absorption in this form was not satisfactory, and a micronized preparation in sucrose syrup was developed for further use. The low solubility of the compound precluded its administration in adequate dose in solution or by intramuscular injection, and it was therefore necessary to give it by mouth.

Methisazone has been used in the prevention and treatment of smallpox and alastrim, and in the treatment of vaccinia infections such as the infective complications of smallpox vaccination.

CLINICAL APPLICATIONS

Prophylaxis of Smallpox. Early work on the effect of isatin 3-thiosemicarbazone in mice infected intracerebrally with vaccinia virus had led to the suggestion (Bauer, 1955) that the compound might be effective prophylactically in persons who had been exposed to smallpox infection. At that time it was not known whether the compound was effective against smallpox as well as vaccinia, and this indeed seemed doubtful in view of its lack of activity in mice infected with ectromelia virus. Subsequent work (Bauer *et al.*, 1962), however, showed that variola major and alastrim viruses were sensitive to isatin 3-thiosemicarbazone and a number of its derivatives, and there was therefore theoretical justification for a trial of the prophylactic effect against smallpox under field conditions. Bauer *et al.* (1963) described the preliminary results of a trial carried out in

Madras. The work was subsequently extended with the object of investigation of the effects of different dosage schedules (Bauer *et al.*, 1969).

A patient who develops smallpox is generally considered to be infectious from the third day of illness onwards. Persons who are associated with the patient thus run a risk of acquiring the infection and developing the disease. Such persons are referred to as *contacts*. A contact who becomes infected remains well for 12 days, during which time the virus is multiplying in the body, probably in the respiratory tract and reticulo-endothelial system. When multiplication has reached a certain point viraemia occurs and clinically manifest illness begins. A smallpox patient who gives rise to an outbreak, or who first draws attention to the presence of the disease, is referred to as an *index* case. Smallpox is an easy disease for carrying out a prophylactic trial, since the illness is conspicuous and can nearly always be diagnosed on clinical grounds alone, and it spreads by case to case contact. Therefore, once index cases have presented themselves it remains only to identify the contacts and to treat them with the drug under investigation. The occurrence of secondary cases must at the same time be compared with that in a comparable group of contacts who are not treated with the compound, in order to establish the natural incidence of secondary cases in the absence of treatment. Susceptibility of the individual contact to smallpox can be inferred from vaccination status. Persons who have never been vaccinated are fully susceptible. Successful primary vaccination or revaccination confers absolute immunity for at least 1 year, and in most cases for longer than this. After 3 years or so immunity begins to decline, and the individual gradually becomes susceptible to infection. It is therefore necessary to record the vaccination status of each contact in order that the results of the trial can be properly interpreted. Furthermore, the distribution of the respective categories of vaccination status, and therefore of susceptibility to smallpox, must not differ significantly between the treated and untreated groups of contacts. It is also desirable that there should not be any significant difference in age distribution, as age is closely correlated with immunity acquired by vaccination.

The groups should also be shown to be equal in respect of degree of exposure; i.e. they should be equal in respect of the chance of getting smallpox, otherwise the incidence of contact cases will not be the same and any conclusions which may be drawn as to the effect of treatment will be invalid. There is no satisfactory way of measuring the degree of exposure. Some idea can be got by recording the day of illness of the index case at the time of admission to hospital. This shows how long the source of infection was present in the house. However, in some contact cases

consideration of the incubation period shows that the infection was not derived from the index case, but from elsewhere in the environment. Nevertheless, the trial must be continued until the distribution of the duration of illness of the index case at the time of admission is the same in the two groups, so that the degree of risk of infection from the most probable source is the same.

Once it is shown that there is no difference between the two groups in susceptibility and presumed degree of exposure, it is valid to assume that any reduction in incidence which occurs in the treated group is due to the treatment, that is, that the drug has been effective.

In conducting the trial several practical points must be attended to. It may be decided to exclude certain types of contact, such as persons who have had smallpox already, or have been vaccinated successfully within recent months, persons who leave the district before the final visit, etc. If the treatment schedule consists of a single dose of the drug, it should be given at the time of the first visit, so that one can be sure that the drug has been taken. If the treatment must be continued for several days it usually becomes impracticable to carry out the number of house visits required unless they are all close together in the same area. Even in this case it is very probable that some of the contacts will be absent when visited. When multiple doses must be given one is therefore obliged to issue the required doses of drug with appropriate instructions, and endeavour to ascertain how much has actually been taken, since the contacts may not necessarily tell the truth. This will introduce bias, but the bias will be against the success of treatment, since some of the contact cases recorded in the treated group will occur in persons who have not taken the full amount of drug, or possibly even none at all. Bias of this type is acceptable, however, since it will tend to give a false negative result. In comparing the results of treatment and no treatment, these people should be retained in the treatment group, since they have received treatment, as far as one knows, but they must be separated off when one wants to compare the prophylactic effect of different dosage schedule since this comparison is not valid unless it is confined to persons who have actually taken the full amount of drug assigned to them. The contacts must also be questioned about side-effects, and one must be prepared to stop the trial if these are unacceptable in severity.

In the trial of the prophylactic effect of methisazone in smallpox carried out in Madras in 1963 the groups of contacts of each index were assigned to treatment or no treatment alternately, but the sequence was not strictly adhered to, and the groups were kept in balance during the progress of

TABLE 19. EFFECT OF METHISAZONE TREATMENT ON THE INCIDENCE OF CONTACT CASES OF SMALLPOX. From Bauer *et al.* (1969)

Group	Treatment	Contacts	Cases	Deaths	Case incidence (%)
1	Completed, all dose levels	2292	6	2	0.26
2	Not completed	318	12	2	3.77
1+2	Total treated	2610	18	4	0.69
3	Not taken	150	11	3	7.33
4	Not offered	2560	102	18	3.99
3+4	Total untreated	2710	113	21	4.17

the trial by periodic adjustment. The drug was given by mouth as a 20% suspension in sucrose syrup, according to 4 dosage schedules. A placebo was not generally used. The gross results were as follows (Table 19). Some or all of the drug issued was taken by 2610 contacts; 18 of these developed smallpox during the observation period and 4 died. The untreated group contained 2710 contacts; 113 of these developed smallpox and 21 died. The case incidence was thus 0.69% in the treated contacts and 4.17% in the untreated contacts. Before claiming that this reduction in incidence has been brought about by the treatment it is necessary to show that the groups do not differ in respect of factors which would affect their liability to contract smallpox.

There are no essential differences in the distribution of vaccination status between the two groups (Table 20), which were therefore equal in susceptibility to infection. The distribution of duration of illness of the index case at the time of the first visit also showed no difference (Table 21). The two groups were therefore equal in their degree of exposure to infection from the index case. The mean duration of illness was around 6 days, which shows how long it takes to recognize a case of smallpox and get the patient into hospital. The analysis also showed that there was little or no infectivity associated with the patient during the first 3 days of illness. This observation is in agreement with generally accepted views based on experimental work on the isolation of smallpox virus from patients in

TABLE 20. DISTRIBUTION OF VACCINATION STATUS. From Bauer *et al.* (1969)

Vaccination status		Incidence of cases			
Before contact	After contact	Treated Groups 1 and 2 combined		Untreated (Group 4 only)	
Not previously vaccinated	Not vaccinated	5/34	(14.71%)	14/25	(56.0%)
	Vaccination failed	3/34	(8.82%)	2/20	(10.0%)
	Vaccination successful	4/161	(2.48%)	25/130	(19.08%)
	Totals	12/229	(5.24%)	41/175	(23.43%)
Previously vaccinated	Not revaccinated	1/232	(0.43%)	7/346	(2.02%)
	Revaccination failed	3/1334	(0.22%)	26/1142	(2.28%)
	Revaccination successful	2/673	(0.30%)	27/751	(3.59%)
	Totals	6/2239	(0.27%)	60/2239	(2.68%)
Records incomplete		0/142†	(—)	1/146†	(0.69%)
Overall total		18/2610	(0.69%)	102/2560	(3.99%)

† These contacts were mostly previously vaccinated adult males who were not seen at the time of the last visit.

various stages of illness. There was also no difference in the distribution of place of residence of the contacts among the 50 municipal divisions of Madras city; heavily infected environments therefore contributed comparable percentages of contacts to each group. This is an important point, since the analysis showed that the bulk of the index cases occurred in only 3 municipal divisions, whereas the cases as a whole were spread over 34 municipal divisions, so that there was a distinct possibility of serious inbalance arising in the risk of acquiring infection from the environment. The groups were also equal in respect of sex and age distribution, in

TABLE 21. DURATION OF ILLNESS OF INDEX CASE ON DAY OF FIRST VISIT. From Bauer *et al.* (1969)

Duration (Days)	Treated			Not Treated		
	No. of contacts	No. of cases	Incidence %	No. of contacts	No. of cases	Incidence
1	0	0	—	3	1	—
2	12	0	—	16	0	—
3	97	0	—	117	0	—
4	209	1	0.48	225	4	1.78
5	445	1	0.22	374	9	2.41
6	417	5	1.20	452	12	2.65
7	400	3	0.75	341	25	7.33
8	337	3	0.89	368	15	4.08
9	119	1	0.84	170	11	6.47
10	237	1	0.42	195	9	4.62
11	34	0	—	52	1	1.92
12	86	0	—	58	6	10.34
13–24	117	0		143	7	4.90
>24	53	0	—	32	1	3.12
Uncertain	47	1	2.13	164	12	7.32
Total	2610	18	0.69	2710	113	4.17

distribution of religions, and in socio-economic status, all factors which could influence the probability of developing smallpox.

Since the groups were comparable in all material respects except treatment, it can be inferred with confidence that the reduction in incidence was due to the treatment, and that methisazone has a prophylactic effect in persons who have been exposed to smallpox infection.

Methisazone was first tried in an adult dose of 3 g twice daily for 4 days. No cases of smallpox occurred in 74 persons who took the course of treatment, whereas there were 9 cases among 132 who received no treatment. The total dosage was then reduced. With a course of 8 doses of 1½ g there was 1 case among 384 contacts, with 2 doses of 3 g there were

no cases among 584, and with a single dose of 3 g there were 5 cases among 1137 contacts. Among the comparable untreated groups there were 105 cases among 2436 contacts. The analysis was restricted to contacts who were considered to have completed the course of treatment assigned to them. The yield of cases among the treated contacts was too low to enable a comparison to be made of the effects of the different dosage schedules. All that one can say is that 2 doses or more gave practically complete protection, and substantial protection was given by a single dose of 3 g. The doses refer in all cases to the amount given to adults, and this amount was reduced proportionally in children and infants.

A small-scale prophylactic trial was also carried out by Rao *et al.* (1969) on family contacts of smallpox patients in Madras. It was confined to persons who had never been vaccinated; the complicating factor of pre-existing immunity was thus removed, but the opportunity of observing the effect of the drug in partially immune persons was thereby lost. Methisazone was given by mouth in a dose of 5 g daily for 3 days, with a proportionate reduction in dose for children of different ages. A placebo was used, and treatment was allocated according to a randomized code. During the observation period 2 cases of smallpox occurred among 17 contacts treated with methisazone, and 8 cases among 19 treated with placebo. Rao *et al.* stated that this difference was not significant, but the value of p by Fisher's exact method is 0.057, a result verging upon significance. It is evident that this trial should have been extended further, since there was the same 4-fold reduction in case-incidence associated with treatment as was observed by Bauer *et al.* (1963).

With the object of confirming the results obtained in Madras, Ribeiro do Valle *et al.* (1965) carried out a trial of methisazone in the prophylaxis of contacts of alastrim in São Paulo, Brazil, in 1964–5. The method was similar to that adopted in Madras, except that alternation of treatment and no treatment between consecutive contacts was strictly adhered to. No placebo was used. Persons who were assigned to treatment but refused it were subsequently evaluated in a separate group which was kept distinct from the controls.

The treatment group included 452 contacts (68 of whom refused treatment) and there were 520 controls. The groups were therefore unbalanced in number to the extent of a 15% excess of controls over treated. This is an interesting example of the inability of methods of treatment allocation to build comparable groups until numbers have become large enough. This would normally create bias in favour of the treatment which would require a subsequent adjustment in the crude data, but there

was one fortunate circumstance about the trial which made this unnecessary. The imbalance occurred almost entirely in persons who had been vaccinated previously, and no cases of alastrim occurred among them. They therefore contributed no information as to the effect of treatment. The further analysis was therefore concentrated on the persons who had not been previously vaccinated. Of these, there were 254 in the treatment group (including 39 who refused treatment) and 267 in the control group. The two groups were therefore now comparable numerically. The analysis according to vaccination status showed that the two groups did not differ in respect of this factor. No information is provided as to the distribution of duration of contact with the index case. Among 215 contacts who took the treatment there were 8 cases of alastrim, compared with 42 cases among 267 in the control group. The case incidence was thus 3.7% and 8.1% respectively. Seven cases occurred among 39 contacts who refused treatment. The figures show that vaccination was usually not carried out after exposure in São Paulo, and the trial was therefore virtually uncomplicated by the factor of pre-existing immunity. Two dosage schedules were used; 2 doses of 3 g were given to 105 contacts, 5 of whom developed alastrim, and 1 dose of 3 g was given to 110, 3 of whom developed alastrim. It is impossible to compare the effect of different dosage schedules on the basis of these figures. The reduction in case incidence between the treated and control groups was in all cases significant at the 5% level or better. The authors conclude that their results confirm the prophylactic effect of methisazone which had been observed in the Madras trial.

Treatment of smallpox. The design of the protocol for a therapeutic trial against smallpox was laid down by Rao, McFadzean and Kamalakshi (1966a). Patients with haemorrhagic smallpox and variola sine eruptione are excluded, and all other patients are given drug or placebo alternately according to admission number. The effect of treatment is assessed according to the following criteria; mortality, and effect on course as assessed by number of patients without fever, mean maximum temperature, and mean time in days to completion of scabbing. When the results have been obtained it is necessary to subdivide the patients according to vaccination status and type of illness. For the latter purpose the classification into flat, ordinary and modified forms as introduced by Rao is used. It is also necessary to subdivide the patients according to duration of illness on the day of admission; no therapeutic effect can be expected in the later stages when the virus has ceased to multiply, but such patients cannot be excluded

from the trial on admission without introducing the possibility of bias. A therapeutic trial against smallpox is thus considerably more complicated than a prophylactic trial, and a large number of patients must be taken into the trial before the various subdivisions become large enough for comparison.

A therapeutic trial of methisazone was carried out by Rao and co-workers in 1964–5. The adult dose was 12 g *statim* and 3 g 6-hourly to a total of 12 doses. The drug was given by mouth. The drug was given to 208 patients and a placebo to 215. The mortalities were 18.6% in the placebo group and 21.6% in the drug group. The only difference in favour of the drug which could be observed was a mortality of 3.6% (3/82) in previously vaccinated patients, compared with 6.5% (6/94) in those given the placebo. The difference was not significant. The mean day of illness on admission was 7.5. Only 25 patients in the placebo group and 24 in the drug group were admitted on the 5th day or earlier, altogether only 11.6% of the total. It was concluded that the drug had no therapeutic effect. For a full evaluation of the effect of treatment it would be necessary to give the drug much earlier in the course of the disease, preferably during the prodromal period or at the first appearance of the rash.

Prophylaxis of smallpox vaccination. Vaccination against smallpox consists of the production of a local infection with vaccinia virus. This leads to the development of a vesicle, which becomes pustular and heals by scabbing. There may be enlargement of the regional lymph nodes and fever, and also oedema and erythema of the skin surrounding the lesion. The severity of this response is usually within acceptable limits, but in adults who have never been vaccinated before, and particularly in elderly persons, the response may be severe enough to make it desirable to have some means of mitigating it. Smallpox vaccination is contraindicated in persons suffering from skin diseases, such as eczema; if these persons are vaccinated they may develop eczema vaccinatum, a generalized eruption of vaccinial lesions accompanied by a severe febrile response which may be fatal in some cases. Such persons are normally excluded from smallpox vaccination, but they may acquire an infection with vaccinia virus through being in contact with vaccinated persons. Also, occasions may arise in which it may be considered necessary to vaccinate them in spite of the contraindication. In such cases it would be desirable to have available a prophylactic agent which would moderate the local lesion and prevent generalization.

A preliminary impression of the effect of methisazone on smallpox vaccination may be obtained from the results of the prophylactic trial

carried out in Madras. Many contacts received primary vaccination or re-vaccination after their exposure to smallpox, and the figures show that these procedures failed somewhat more often in those contacts who were treated with methisazone. Primary vaccination failed in 21 of 152 untreated contacts (13.82%) and in 35 of 196 treated contacts (17.86%); although the failure rate was higher in the treated group the difference was not significant ($p > 0.3$). A similar increase in the failure rate associated with treatment occurred with revaccination. This failed in 1143 of 1890 untreated contacts (60.48%) and 1335 of 2033 treated contacts (65.67%). This difference was highly significant ($p < 0.001$). In this trial only success or failure of vaccination was recorded and no attempt was made to measure the size of the lesions, and it may be concluded that methisazone caused a slight but definite inhibition of the vaccination reaction which was sufficient to prevent a take in some cases.

A similar effect was noted in the Brazil trial by Ribeiro do Valle *et al.* Primary vaccination failed in 30 of 48 untreated contacts (61.25%) and 20 of 28 contacts treated with methisazone (71.5%); with revaccination the failure rates were 19 of 23 (82.6%) and 9 of 9 (100%) respectively. The differences, however, were not significant ($p > 0.25$).

The effect of methisazone on smallpox vaccination has also been studied experimentally. Landsman and Grist (1964) vaccinated 32 student volunteers, and after 2 days 16 were treated with methisazone. The intended course was 3 g twice daily for 3 days, but owing to vomiting the course was not completed in many cases. The mean diameter of the lesions was 0.42 cm in the treated group and 0.59 cm in the remainder. Significant rises in titre of inhibiting antibody occurred in 4 of 15 treated and 10 of 16 untreated subjects. In spite of the smallness of the groups it was considered that methisazone had had some inhibiting effect upon the response to vaccination.

Herrlich, Stickl and Munz (1965) carried out primary vaccination on 51 children ranging in age from 4 months to 2¾ years, and 33 were treated with methisazone for 3 days. The latter were divided into two groups; 15 received a daily dose of 25 mg/kg and 18 received 100 mg/kg daily in 2 divided doses. The 2 dosage groups were subdivided into 3 further groups, in which treatment was begun 1 day before vaccination, on the same day, and after 2 days respectively. The vaccination lesions were not measured but were graded qualitatively according to severity. No effect of treatment upon the lesion could be detected. This experimental design is open to criticism on two counts. A dose of 25 mg/kg seems too low to be effective, and the treatment was in most cases given too early in the

incubation period. The initial course of the infection may have been retarded, but residual virus would continue to grow at the normal rate after treatment had been discontinued, and the final size of the lesions would therefore be unaffected. Further work was carried out with rabbits, which were infected with vaccinia virus on the skin and given methisazone by mouth in doses ranging up to 300 mg/kg. The results were negative, but here again treatment was confined to the first 3 days after infection.

Jaroszyńska-Weinberger and Mészáros (1966) studied the effect of methisazone on primary vaccination in 26 children for whom smallpox vaccination was contraindicated on account of skin disease, age and other reasons. The drug was given by mouth in an initial dose of 100 mg/kg, followed by 50–60 mg/kg daily for 3–6 days; treatment was started on the 4th day after vaccination. In view of the risk of vaccinating such children there was no control group, and the effect of methisazone was compared with that obtained in a group of 29 similar children who received primary vaccination under the protection of antivaccinial gamma-globulin. The dimensions of the local lesion were markedly reduced in the methisazone group, and the difference was highly significant ($p = 0.005$); since antivaccinial gamma-globulin itself is known to reduce the severity of the vaccination lesion it can be inferred that a highly significant effect would have been obtained with methisazone if a true control group had been used. The titres of inhibiting antibody were determined at intervals up to 9 weeks after vaccination in 19 children treated with methisazone and 22 treated with gamma-globulin. A rise of titre occurred in all cases; the geometric mean titres in the two groups were 172.1 and 193 respectively and the difference was not significant ($p > 0.5$). These results do not support those of Landsman and Grist, but the latter workers were using a true control group; nevertheless, it is evident that satisfactory immunity develops in patients treated with either methisazone or gamma-globulin. No complications of any importance occurred.

The results as a whole tend to show that methisazone will mitigate the severity of the vaccination lesion; there is therefore some indication for using it for this purpose. Although the number of cases treated by Jaroszyńska-Weinberger and Mészáros was small, the absence of serious complications suggests that children with skin complications may be vaccinated if it is absolutely essential but on grounds of general caution it would seem better to cover the procedure with both methisazone and antivaccinial gamma-globulin.

Bondarev *et al.* (1970) studied the course of primary smallpox vaccination in 110 children treated with methisazone in comparison with a control

group of 52 children who were left untreated. The children were aged 2–4 years, and primary smallpox vaccination had been delayed on account of illness, previous failure of vaccination, exudative diatheses and other conditions. Methisazone was given by mouth as tablets in a dose of 10 mg/kg twice daily, and treatment was started at various times after vaccination. Local and general reactions to vaccination were milder in the treated group; there were 17 mild reactions among 20 children treated for the 4 days following vaccination, compared with 26 of 52 in the control group. Reactions of moderate severity occurred in the 3 remaining treated children and in 24 of the control group. The differences in favour of the treatment were highly significant ($p < 0.02$). The duration of the febrile period was also significantly shorter in the treated group ($p < 0.05$), and the area of the local lesion was smaller ($p < 0.05$). The drug was well tolerated, and vomiting occurred only once in 1 child. The formation of neutralizing antibody was not depressed by the treatment. This work is of particular interest in view of the good result obtained with doses much smaller than those used by other investigators, and it would be highly desirable to carry out a dose-finding study in order to establish the minimum dose which will achieve the degree of protection desired.

Treatment of eczema vaccinatum. If smallpox vaccination is carried out on persons suffering from eczema, or who have had eczema in the past, the local lesion develops into a progressive infection in which a secondary eruption of vesicles and pustules appears, which may become so extensive as to cover the whole body. There is also a severe general reaction with high fever and prostration, and the condition closely resembles smallpox. The mortality is considerable, and has been reported as being as high as 30%. The eruption is concentrated upon the eczematous areas, particularly the face, and the general appearance of a typical case is illustrated in Fig. 14.

The clinical course of eczema vaccinatum is very variable, and estimates of therapeutic effect have so far been based upon clinical impression.

The successful treatment of eczema vaccinatum with methisazone was first described by Turner, Bauer and Nimmo-Smith (1962). The patient was a boy aged 7 months who had suffered from infantile eczema up to the age of 4½ months. He contracted an accidental vaccinia infection from his parents, who had been recently vaccinated. Lesions appeared all over the scalp, becoming almost confluent, and spread to the face, shoulders and upper and lower limbs. The general condition was very severe, with pyrexia, prostration and toxaemia. There was no response to vaccinial

hyperimmune gamma-globulin, but the condition cleared up rapidly after treatment with methisazone given orally in a dose of 250 mg every 6 hr.

An analysis of 22 cases of eczema vaccinatum treated with methisazone in varying doses has been given by Bauer (1965a). The effect of treatment was assessed from clinical impression and mortality, and was subdivided into 3 groups. It was considered to have been effective in 12 patients, all of whom recovered. Four had failed to respond to antivaccinial gamma-globulin. The effect was considered to be doubtful in 7 patients, 1 of whom died; 2 of these had failed to respond to gamma-globulin. Treatment was ineffective in 4 patients, 3 of whom died. The mean initial and total doses were 152 and 869 mg/kg in the first group, 63 and 324 mg/kg in the second, and 48 and 231 mg/kg in the third group. The duration of treatment ranged from 1½ to 7 days. From these observations an effective dosage schedule was devised for further use, consisting of an initial dose of 200 mg/kg, followed by 8 doses of 50 mg/kg at intervals of 6 hr, to make a total dose of 600 mg/kg.

Mainwaring (1962) treated a severe case of eczema vaccinatum with methisazone; rapid recovery took place, but it is difficult to assess the effects of treatment in this case as other therapeutic measures were also used. Successful results have been reported by Webb, Marks and Reed (1965), Marsh and Mitchell (1965), Adels and Oppé (1966) and Jaroszyńska-Weinberger (1970).

Treatment of vaccinia gangrenosa. The lesion produced by smallpox vaccination normally heals with the development of immunity, but if the immune response is defective for any reason the lesion will show no tendency to heal, but will enlarge indefinitely. Satellite lesions may appear round it, and metastatic lesions in any part of the body. These lesions will enlarge in their turn, giving rise to destruction of skin which becomes so extensive that the patient dies. This condition is known as vaccinia gangrenosa, and is invariably fatal in the absence of treatment. Two types may be distinguished. The first occurs in infants with agammaglobulinaemia or hypogammaglobulinaemia, and vaccinia gangrenosa arising after smallpox vaccination may be the first evidence of the immunological defect. The second type occurs in patients of any age who are receiving immunosuppressive and cytotoxic drugs for the treatment of malignant conditions. Cure may be obtained with antivaccinial gamma-globulin (Kempe, 1960), but many cases fail to respond. Recovery from the virus infection is usually followed by eventual death from the underlying disease condition.

The results obtained in the treatment of 10 cases of vaccinia gangrenosa

with methisazone have been analysed by Bauer (1965a). Five patients recovered from the vaccinial infection; 2 of these had failed to improve after antivaccinial gamma-globulin, and in 1 the response could not be assessed. The mean initial dose of methisazone was 116 mg/kg and the mean total dose was 638 mg/kg. Of the 5 patients who died, 2 had received no loading dose, and in the remainder it was probably too low. The mean initial dose in the 5 fatal cases was 84 mg/kg, and the mean total dose 791 mg/kg. They included the cases reported by Connolly, Dick and Field (1962), Flewett and Ker (1963) and White (1963). Abrassart and Daemers (1967) also failed to observe any effect in an infant aged 3 months treated with methisazone in a dose of 1 g daily, who had also failed to respond to antivaccinial gamma-globulin. Davidson and Hayhoe (1962) reported a favourable effect in a patient suffering from leukaemia who was vaccinated as a contact of a suspected case of smallpox. Vaccinia gangrenosa developed, with metastatic lesions in various parts of the body. There was no response to antivaccinial gamma-globulin. Methisazone was given in a daily dose of 5 g for 7 days, and the lesions healed with scab formation. The patient subsequently died of the underlying disease. Similar favourable results had been reported by Van Rooyen *et al.* (1967) in a woman with unrecognized immunological deficiency who had been vaccinated in a misguided attempt to cure alleged herpetic ulcers of the mouth, and by Brainerd, Hanna and Jawetz (1967) in a woman suffering from Hodgkin's disease. Another favourable result is shown in Fig. 15, which is taken from Hansson, Johansson and Vahlquist (1966). Jaroszyńska-Weinberger (1970) also obtained cures in 2 children with vaccinia gangrenosa in its localized stage. Kempe *et al.* (1967) obtained virological cure in 5 of 9 patients with extensive vaccinia gangrenosa which had failed to respond to immunotherapy. It is evident from published case reports that cure may be obtained in one-half of patients with vaccinia gangrenosa, whereas the remainder fail to respond to treatment with antivaccinial gamma-globulin or methisazone. Kempe suggests that favourable results can be obtained in patients with a single immunological defect (Bruton-type agammaglobulinaemia) or with defects acquired as a result of lymphoma, leukaemia and other reticuloses, whereas treatment is ineffective in the presence of multiple defects (Swiss-type agammaglobulinaemia).

As a result of experience gained so far the dosage schedule recommended for the treatment of vaccinia gangrenosa is the same as for eczema vaccinatum, a loading dose of 200 mg/kg followed by 8 doses of 50 mg/kg at intervals of 6 hr.

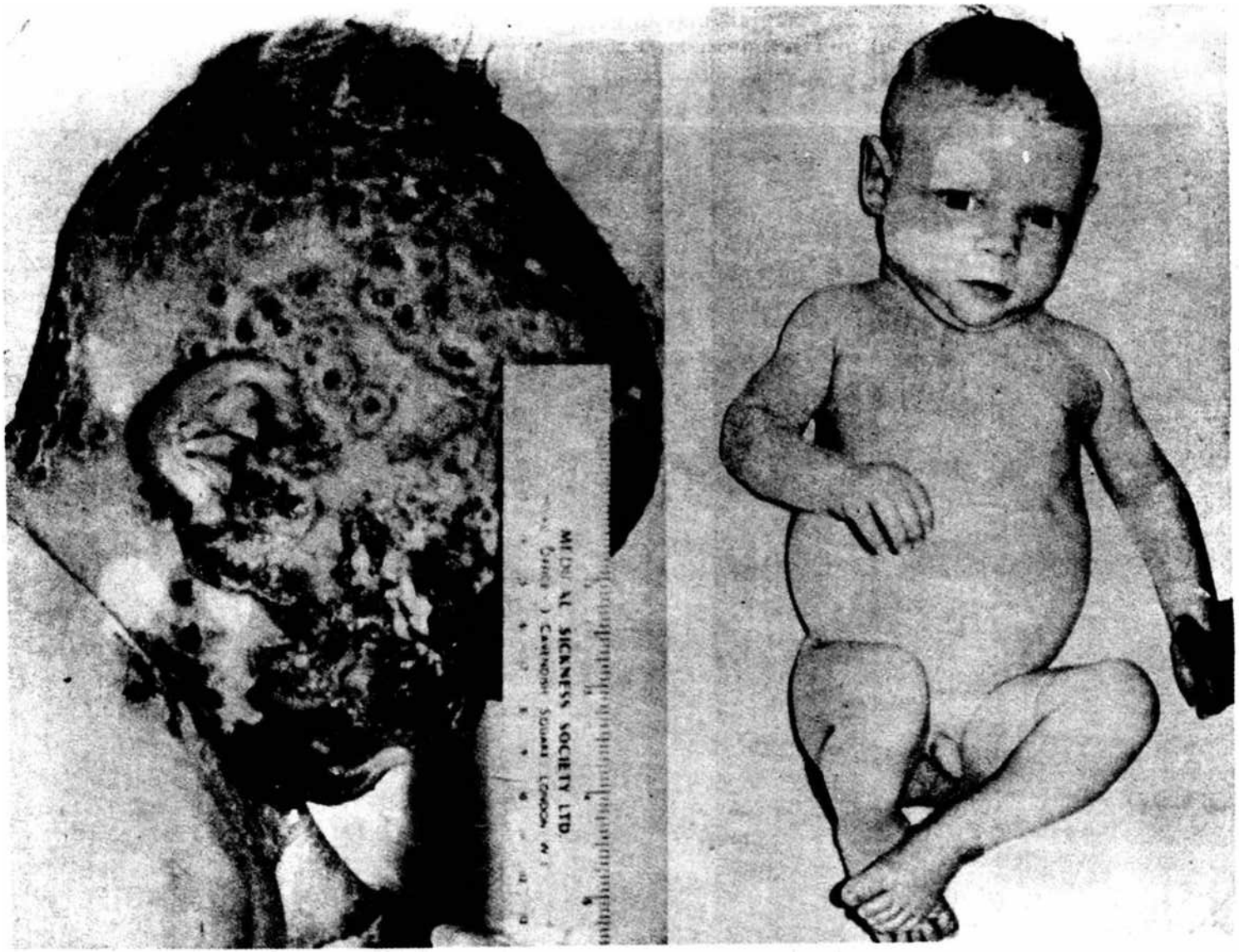


FIG. 14. Eczema vaccinatum. *a*, 7th day of rash; the eruption closely resembles that of smallpox; *b*, recovery after treatment with methisazone and antibiotics (case reported by Mainwaring, 1962).

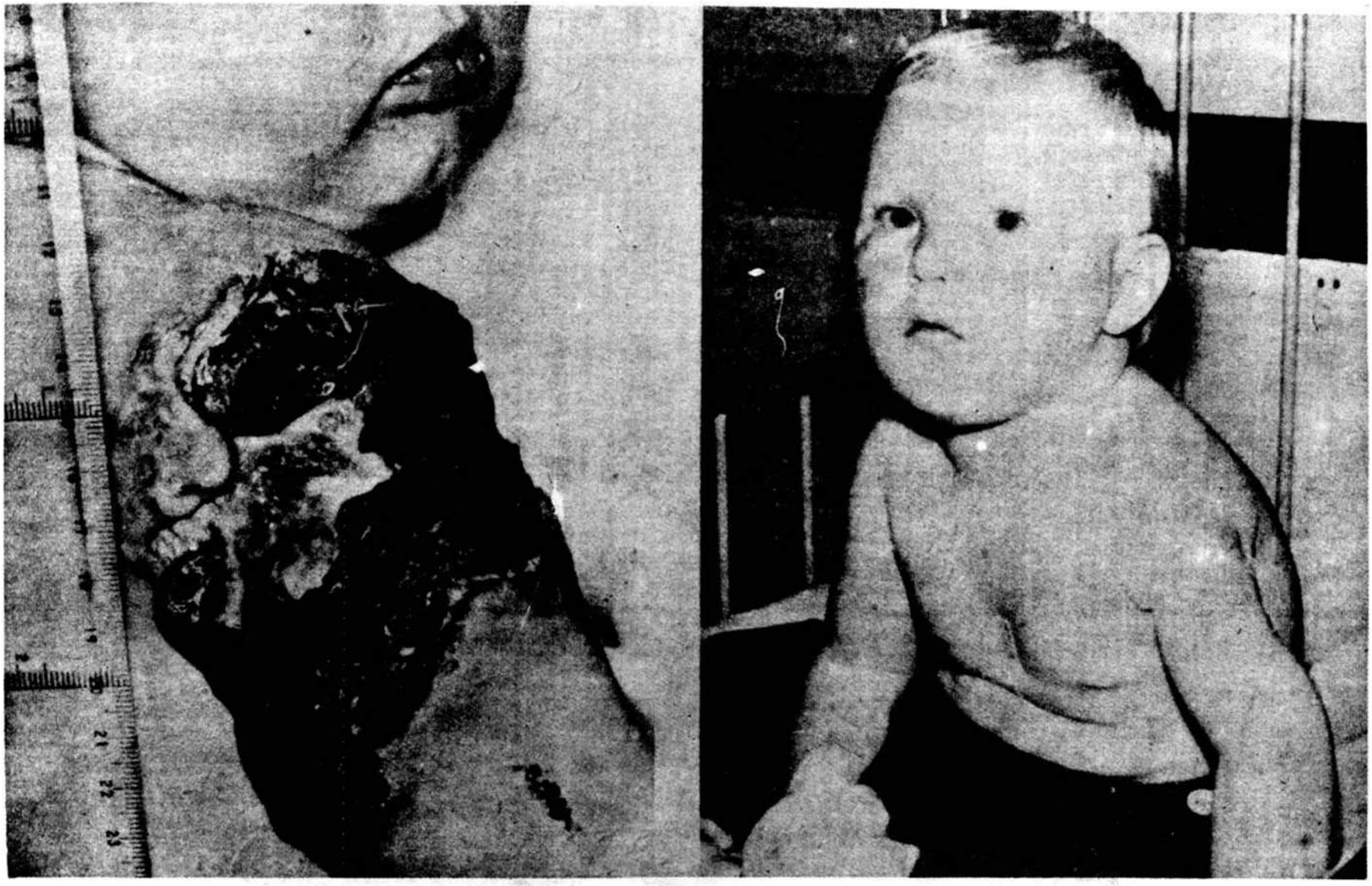


FIG. 15. Vaccinia gangrenosa. *a*, appearance of lesions 5½ months after vaccination; *b*, condition on discharge after treatment with methisazone and closure of defect by skin grafting.

Side-effects of methisazone. The oral administration of methisazone is frequently accompanied by nausea and vomiting. This occurred in 16.7 to 27.3% of persons treated in Madras, and in 66% of persons treated in Brazil. This variation may be due to differences in race and diet. Landsman and Grist (1964) observed severe vomiting in student volunteers who took a course of methisazone, but they used a preparation of the drug which was not standard material, and no such incidents have been reported since this time. Herrlich, Stickl and Munz (1964) observed vomiting in 17 of 33 children; Jaroszyńska-Weinberger and Mészáros (1966) observed vomiting in 10 of 26 children, but they commented that the vomiting was in no way as severe as that reported by Landsman and Grist.

There have been conflicting reports on the efficacy of the standard anti-emetic drugs in suppressing the vomiting. Ferguson (1964) considered that vomiting could be reduced by the previous administration of cyclizine, but Ribeiro do Valle *et al.* (1965) found cyclizine ineffective, and chlorpromazine and cyclizine were found ineffective in the Madras trial. Vomiting is acceptable as a side-effect when the drug is used in the prophylaxis of a dangerous infection, but it has the disadvantage of causing the loss of some or all of the drug administered, and therefore a reduction in or loss of the prophylactic effect.

Pharmacology of methisazone. A method for determining the concentration of methisazone in blood samples has been described by Turner, Bauer and Nimmo-Smith (1962). Heparinized venous blood is acidified with 1/10 volume of 2 N NaOH and shaken with an equal volume of toluene. Methisazone is thereby converted into the acid form which is soluble in toluene, and its concentration in the toluene layer can be determined by measuring the optical density at 375 mμ. The concentration can be calculated by reference to the optical density of a toluene solution of known concentration. The method can be made somewhat more sensitive by extracting the toluene solution into N NaOH and reading the optical density at 400 mμ. This procedure converts methisazone into the sodium salt, and the peak of absorption shifts to 400 mμ. For the determination of methisazone in faeces, an aliquot is freeze-dried and refluxed for 1 hr with 20 parts of benzene. An aliquot of the benzene solution is then extracted into N NaOH and the optical density is measured at 400 mμ.

Turner *et al.* found a plasma concentration of 1.9 μg/ml (8 parts) in a sample taken from a child on the second day of treatment with methisazone.

Using the same method, Kempe, Rodgerson and Sieber (1965) found

maximum levels 4–7 hr after oral administration. In an infant with progressive vaccinia a single oral dose of 200 mg/kg gave a maximum level of 20 μg/ml (85.5 μM), and a dose of 100 mg/kg gave a maximum of 12 μg/ml (51.3 μM). In an adult with eczema vaccinatum a single dose of 3 g gave a maximum level of 6.3 μg/ml (27.0 μM). The drug had mostly disappeared after 10–12 hr, and multiple dosing did not cause any accumulation. Gomez and Sandeman (1966) found much lower levels in a small series of patients with various malignant conditions.

There is no information available on the levels of methisazone retained in the skin and internal organs.

HERPES VIRUS (HERPES SIMPLEX)

Isatin 3-thiosemicarbazone given subcutaneously in repeated doses of 100–125 mg/kg had no effect in mice infected intracerebrally with herpes virus (Bauer and Sadler, 1960a), but this cannot be taken as a definite negative result as it is not known whether the compound can cross the blood–brain barrier. Rapp (1964) found that methisazone in concentrations up to 1 μg/ml (4.8 μM) did not inhibit plaque formation by herpes virus on monolayers of rabbit kidney fibroblasts. This again cannot be taken as a negative result in view of the low maximum concentration employed; higher concentrations could not be tested as the compound precipitated when added to the overlay medium. Caunt (1967) found that methisazone reduced the yield of herpes virus from cultures of human thyroid cells, and with a concentration of 20 μM the inhibition approached 100%. Herrmann (1968) obtained a zone of inhibition with methisazone in plaque inhibition tests carried out with herpes virus growing in HeLa cells. In the clinical field, Hutfield and Csonka (1964) found that methisazone given by mouth in 3 doses of 1 g per day for 2 days had no effect upon the duration of the lesions in 6 patients suffering from herpes genitalis.

In summary, it seems possible that methisazone will inhibit the multiplication of herpes virus in some cell systems but not in others, but the inhibition obtained is not great enough to be of clinical interest. It is also possible that chemotherapeutic sensitivity will depend on the type of virus. Most of the studies reported were carried out with untyped strains.

VARICELLA—ZOSTER VIRUS

Rapp (1964) found that methisazone in a concentration of 1 μg/ml did not reduce the number of plaques formed by zoster virus in monolayers

of human embryo lung cells. Caunt (1967) studied the effect of methisazone on the growth of varicella virus in primary cultures of human thyroid cells. In this tissue culture system varicella virus can be liberated by sonication and titrated in the same cells. In preliminary experiments it was shown that methisazone in a concentration of 20 μM did not cause any significant reduction in cell numbers over a period of 48 hr; when the cells were transferred to medium without methisazone and incubated for 24–48 hr they were still capable of supporting the growth of varicella virus to full titre. It was concluded that the compound was not toxic for human thyroid cells in a concentration of 20 μM. In some cases the cells appeared to be unaffected after exposure to concentrations of 30 and 40 μM. In infected cultures varicella virus appeared after 24 hr and reached a maximum titre of nearly 10^4 pfu/ml after 72 hr. In cultures containing methisazone in a concentration of 20 μM the amount of virus produced was lower by about 1 log unit. A dose–response curve of percentage inhibition against methisazone could be obtained, and in some experiments 50% inhibition could be obtained with a concentration of methisazone as low as 5 μM. Caunt was using a much more satisfactory technique than Rapp, and the antiviral activity of methisazone against varicella virus seems to have been demonstrated beyond doubt.

There are as yet no reports of the use of methisazone in the treatment of varicella or zoster in man. Reed *et al.* (1966) carried out a small trial of the prophylactic effect of methisazone in susceptible children exposed to chickenpox. No protection could be observed.

INFECTIOUS BOVINE RHINOTRACHEITIS

Munro and Sabina (1970) have investigated the effect of methisazone and isatin 3-thiosemicarbazone on the multiplication of infectious bovine rhinotracheitis virus, a member of the herpes virus group. Cultures of the MDBK line of bovine kidney cells were infected with virus in a multiplicity of six plaque-forming units per cell. After incubation at 37°C for 1 hr to permit adsorption of virus, the cultures were washed and covered with medium containing methisazone in concentrations ranging up to 20 μg/ml (82 μM). After incubation for 24 hr the yield of virus in each culture was determined by plaque counting on monolayers of the same cells. The compound in a concentration of 5 μg/ml reduced the yield by about 1 log unit in comparison with untreated control cultures, and a reduction of 3 log units was obtained with 10 μg/ml and 4–5 log units with 20 μg/ml. Five strains of the virus were used, and were found to

vary somewhat in their sensitivity to inhibition. In similar experiments with isatin 3-thiosemicarbazone a considerably greater antiviral effect was observed, and a concentration of 4 μg/ml gave a reduction in yield up to 3 log units. This is in contrast to the activity observed with vaccinia virus, where methisazone has twice the activity of isatin 3-thiosemicarbazone.

Experiments on the mode of action were carried out with isatin 3-thiosemicarbazone alone. The compound inhibited the synthesis of virus RNA and DNA, but did not affect protein synthesis. The mode of action was thus not the same as against the poxviruses, where the compound causes instability of late messenger RNA without impeding its production, leading therefore to inhibition of protein synthesis.

POLIOMYELITIS VIRUS

Lwoff and Lwoff (1964) found that the multiplication of a wild strain of type 1 poliomyelitis virus was inhibited by isatin 3-thiosemicarbazone in a concentration of 40 μM. The wild strain was inhibited by guanidine; a mutant which was resistant to guanidine was less sensitive to inhibition by isatin 3-thiosemicarbazone. A mutant was obtained which required the presence of guanidine for normal multiplication; with this mutant the addition of isatin 3-thiosemicarbazone increased the yield of virus.

These results show that wild type 1 poliomyelitis virus is inhibited by isatin 3-thiosemicarbazone almost to the same extent as vaccinia virus. The inhibition can be easily demonstrated by plaque inhibition tests, and all three types of virus are equally sensitive. A result obtained with the type 1 attenuated Sabin strain in HeLa cells is shown in Fig. 16 (Bauer, Apostolov and Selway, 1970).

FOOT-AND-MOUTH DISEASE VIRUS

Polatnick (1965a, b) investigated the effect of isatin 3-thiosemicarbazone and methisazone on the growth of type A foot-and-mouth disease virus in cultures of calf kidney cells. In a preliminary study of toxicity it was found that the glucose and oxygen consumption of the cultures was unaffected by the presence of either compound in the medium in a concentration of 100 μm/ml over a period of 5 hr. Cells incubated for 24 hr with the same concentrations remained attached to the glass. It was concluded that the compounds were not toxic at a concentration of 100 μg/ml. This figure is greater than the water solubility of the compound at

FIG. 16. Activity of methisazone against type 1 poliomyelitis virus. A monolayer of HeLa cells was infected with the virus and covered with an agar overlay. The disc contains 10 μg of the compound. It is surrounded by a narrow zone of toxicity, beyond which there is a zone in which the development of plaques has been inhibited (Bauer *et al.*, 1970).

37°C; the method of dissolving the compounds is not stated, and it is possible that a considerable proportion was present in the form of finely suspended solid. Cultures of primary calf kidney cells were treated with a solution of the compounds, and after 2 hr the solution was removed and foot-and-mouth disease virus was added in a multiplicity of 3–5 pfu per cell. The virus was removed after 30 min and the test compound was added, dissolved in a maintenance medium consisting of Hanks salt solution in 1 % pH 7.5 tris buffer containing 0.1 % glucose. After incubation for 5 hr the cells were lysed with 1 % sodium laurylsulphate and the amount of virus released was determined by plaque titration. In the presence of isatin 3-thiosemicarbazone in a concentration of 50 μg/ml the yield of virus was 6.5 log units/ml, compared with 7.1 in control cultures to which the compound had not been added; with 100 μg/ml the yield was 5.6 log units. Methisazone caused an even greater degree of inhibition; the yield was 5.4 log units with a concentration of 10 μg/ml and 4.4 log units with 50 μg/ml. When the tests were repeated in complete growth medium containing lactalbumin hydrolysate and bovine serum the compounds were somewhat less effective in inhibiting virus multiplication. The virus did not lose any infectivity when incubated for 3 hr at 37°C with the compounds in a concentration of 200 μg/ml, and the antiviral effect could therefore not have been due to extracellular inactivation of the virus. Methisazone was effective only when added to the cultures during the first 2 hr of the growth cycle; no inhibition was observed when the compound was added after 3 hr or later. It was concluded that methisazone might be worth trying against foot-and-mouth disease in cattle, but there are as yet no reports of this having been done.

ADENOVIRUSES

Methisazone has been found to inhibit the multiplication of a number of adenoviruses in HeLa cells (Bauer and Apostolov, 1966). Cultures were infected with adenovirus type 11 at a multiplicity of around 4, and culture medium containing methisazone was added. Incubation was carried out for 42 hr; the virus present in the cells was liberated by freezing and thawing or sonication and titrated as haemagglutinin with patas monkey red cells. In the presence of 30 or 40 μM methisazone the production of virus was completely inhibited, whereas the titre of haemagglutinin in control cultures not treated with the compound was 512–1024. A similar inhibition of virus multiplication was observed when the yield was determined by infectivity titration. Titration of untreated cultures sampled at intervals

after infection with adenovirus 11 showed that new virus appeared after 10 hr and attained a maximum titre of around 8192 after 30–40 hr. In similar cultures treated with 30 μM methisazone the formation of new virus was completely inhibited. Inhibition was still complete when the addition of methisazone was delayed until 13 hr after infection, but increasing yields of virus were obtained when the addition of the compound was delayed beyond this time.

In tests of toxicity it was found that HeLa cells retained normal morphology when cultured for 48 hr in the presence of 40 μM methisazone; they grew normally when subsequently subcultured. Cells incubated with 40 μM methisazone for 2 hr at 37°C and then washed were still able to support the multiplication of virus to full titre when subsequently infected. It was therefore concluded that the suppression of virus multiplication observed was a true antiviral effect, and was not due to a toxic effect upon the cell cultures.

Inhibition of virus multiplication was also observed in similar experiments with adenoviruses of types 3, 7, 9, 14, 16, 17, 21 and 28, and also SV15, a simian adenovirus.

The structure–activity relationships of methisazone against the adenoviruses were similar to those already established for vaccinia. Activity was abolished by replacing the sulphur in the side-chain with oxygen or by alkylation in the 4′-position, and reduced by dealkylation in the 1-position or substitution in the aromatic ring.

There are as yet no reports of the use of methisazone in the prevention or treatment of adenovirus infections in man.

ARBOVIRUSES

According to early reports published, the antiviral activity of the thiosemicarbazones did not extend to the arboviruses. Minton, Officer and Thompson (1953) found no activity in mice infected with Rift Valley fever virus and treated with isatin 3-thiosemicarbazone. Bauer and Sadler (1960a) found that the same compound had no detectable activity in mice infected intracerebrally with Ilhèus, Wyeomyia, Zika, California, Ntaya, Semliki Forest, dengue 1, Anopheles A and Anopheles B viruses. These findings are not definitely negative, however, since it is not known whether the compound can cross the blood–brain barrier in adequate amounts.

More recently, Bauer, Apostolov and Selway (1970) have shown that the multiplication of Bunyamwera and Semliki Forest viruses in HeLa and patas monkey kidney cells was inhibited by methisazone to an extent

which was proportional to the concentration of compound in the medium. The multiplication of Sindbis virus was not affected, and it was thus concluded that the inhibition seen with Bunyamwera and Semliki Forest viruses was a true antiviral effect, and was not due to a toxic effect of the compound upon the tissue cultures.

RHINOVIRUSES

The effect of isatin 3-thiosemicarbazone, methisazone and certain congeners on rhinoviruses was investigated by Gladych *et al.* (1969). The viruses were grown in human diploid cells in medium containing the compounds in various concentrations, and the antiviral activity was assessed from the degree of inhibition of the cytopathic effect. The 1059 strain of rhinovirus was inhibited by isatin 3-thiosemicarbazone in a concentration of 20 μg/ml. Methisazone inhibited this strain in a concentration of 11 μg/ml, and also inhibited the 33342 strain (21 μg/ml) and the HGP strain (22 μg/ml). Tricyclic derivatives formed by closure of the side-chain were also active.

TABLE 22. ANTIVIRAL SPECTRUM OF METHISAZONE AND ANALOGUES. From Bauer *et al.* (1970)

Nucleic acid	Group	Virus
DNA	Poxvirus	Vaccinia
		Smallpox
		Cowpox
	Adenovirus	Adenovirus
	Herpesvirus	Varicella
		Herpes?
RNA	Picornavirus	Poliomyelitis
		Echovirus
		Rhinovirus
		FMD
	Reovirus	Reovirus 3
	Arbovirus	Rift Valley Fever
		Bunyamwera
		Semliki
	Myxovirus	Influenza A, B
	Paramyxovirus	Parainfluenza 1

OTHER VIRUSES

Bauer, Apostolov and Selway (1970) found that methisazone inhibited the multiplication of types 3, 7, 11, 12, 13 echovirus, reovirus type 3, the A/England/66 strain of influenza virus and the Sendai strain of parainfluenza 1 virus in appropriate tissue culture systems.

The full spectrum of antiviral activity of methisazone and related compounds is shown in Table 22.

OTHER THIOSEMICARBAZONES

M AND B 7714

Antiviral activity is not confined to thiosemicarbazones of the benzaldehyde and isatin series. Slack *et al.* (1964) studied the new monocyclic ring system 1,2-thiazole (*iso*thiazole), and found that one of its derivatives, 3-methyl-4-bromo-5-formyl*iso*thiazole thiosemicarbazone, was active against vaccinia virus. The compound was subsequently known as M and B 7714, and its structural formula is shown in Fig. 17.

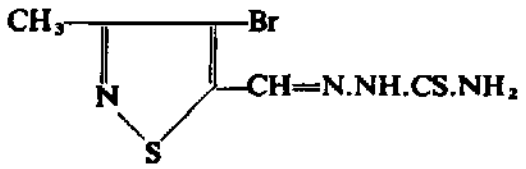


FIG. 17. Structural formula of 3-methyl-4-bromo-5-formyl*iso*thiazole thiosemicarbazone (M and B 7714).

ACTION AGAINST VACCINIA VIRUS

Mice were infected intracerebrally with vaccinia virus and given M and B 7714 by mouth once daily for 4 days in doses of 120, 250 and 500 mg/kg. Both the survival rate and the survival time of animals which died were markedly increased in comparison with control animals which were not treated (Table 23).

A protective effect was still observed when treatment was delayed up to 3 days after infection. The animals which survived were immune to challenge infection with 10^5 LD_{50} of virus. When mice were inoculated on the tail with vaccinia virus the development of the lesions could be prevented by the oral administration of M and B 7714 for 4 days in a daily dose of 100 mg/kg. The formation of plaques in mouse embryo

TABLE 23. EFFECT OF M AND B 7714 AGAINST VACCINIA IN MICE.
From Slack *et al.* (1964)

Dose (mg/kg)	Mean survival time (days)	No. surviving at 10 days
500	9.4	22/30
250	9.0	18/29
120	8.0	12/29
Control	4.6	0/30

fibroblast monolayers of neurovaccinia virus was completely suppressed when the compound was present in the medium in a concentration of 5.6 μg/ml, and some inhibition of plaque formation occurred with a concentration of 0.5 μg/ml.

RABBITPOX VIRUS

M and B 7714 was effective against rabbitpox infection in rabbits. After intranasal infection with the Utrecht strain of the virus, 4 of 5 rabbits survived after oral treatment with 100 mg/kg daily for 4 days, whereas there were no survivors among 5 control animals infected similarly but not treated with the compound.

SMALLPOX VIRUS

In 2-day-old mice infected intracerebrally with the Higgins strain of variola virus and treated with the compound in a daily dose of 1 mg, the maximum titre of virus attained in the brain was around 2 log units lower than the titre in untreated control animals, and most of the treated animals survived the infection.

OTHER POX VIRUSES

M and B 7714 had no effect in sheep infected with contagious pustular dermatitis virus, mice infected with ectromelia, or chickens infected with fowlpox virus.

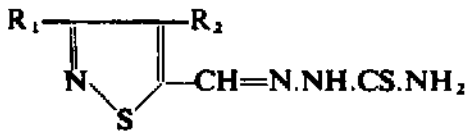
OTHER VIRUSES

The compound was not active in mice infected with influenza, Rift Valley fever or Columbia SK viruses.

STRUCTURE–ACTIVITY RELATIONSHIPS

The structure–activity relationships of M and B 7714 and related compounds were studied by Caton *et al.* (1965), and are shown in Table 24. The compounds were administered by mouth once a day for 4 days to mice infected intracerebrally with the IHD strain of neurovaccinia virus, and survival was recorded in comparison with control animals which did not receive treatment. The activity of the compounds was not assayed but was expressed in arbitrary terms.

TABLE 24. STRUCTURE–ACTIVITY RELATIONSHIPS OF 5-FORMYL*iso*THIAZOLE THIOSEMICARBAZONE. From Caton *et al.* (1965)



R_1	R_2	Daily dose (mg/kg)	Mean survival time (days)		Activity
			Treated	Controls	
H	H	120	9.3	5.3	++
CH_3	H	150	9.0	5.4	++
H	CH_3	70	10.0	5.3	++
H	Cl	120	10.8	5.0	+++
H	Br	1000	11.5	5.0	+++
H	I	500	10.0	4.9	+++
CH_3	Cl	1000	12.2	6.0	+++
CH_3	Br	1000	12.7	4.8	+++†
CH_3	I	1000	10.3	6.0	++

† M and B 7714

The activity was markedly dependent upon the position of the formylthiosemicarbazone side-chain; when this was in the 4-position the activity was reduced and the 2-formylthiosemicarbazone was inactive. In the

5-formyl*iso*thiazole thiosemicarbazones, substitution by halogen in the 4-position increased the antiviral activity and also reduced the toxicity; the 4-bromo derivative was particularly effective in this respect. Substitution of a methyl group in the 3-position reduced toxicity without reducing activity. Combination of the two substitutions gave the 3-methyl-4-chloro and 3-methyl-4-bromo derivatives, which had high activity and low toxicity. The latter derivative is M and B 7714. Substitution in the side-chain generally led to a loss of activity. The activity was also reduced or abolished if the side-chain was cyclized to form a mercaptotriazole or aminothiadiazole.

A notable feature of the active compounds was that their activity was much greater when given by mouth than when given subcutaneously.

PROPHYLAXIS OF SMALLPOX

The prophylactic effect of M and B 7714 in persons exposed to smallpox infection was investigated by Rao *et al.* (1966b). The trial was carried out among the house contacts of patients admitted with a diagnosis of smallpox to the Infectious Diseases Hospital, Madras, and was restricted to persons who had never been vaccinated. Treatment with drug or placebo was offered according to a previously prepared randomized schedule. The drug was given by mouth as a 20% suspension; adults received 4 g daily on 3 successive days, and the dose was reduced proportionately for children and infants. The trial was started with the intention of carrying it out under double-blind conditions, but this proved impossible since the occurrence of side-effects made it possible to deduce which persons had received the drug. The contacts were observed over a period of 16 days to see whether they developed smallpox. Of 201 contacts treated with placebo, 60 (29.9%) developed smallpox during the period of observation, compared with 40 of 196 (20.4%) who had been treated with M and B 7714. The reduction in incidence associated with treatment with the drug was significant at the 5% level. The drug and placebo groups were similar in regard to age-distribution and presumed duration of exposure to infection. There were 12 deaths (20.0%) among the patients of the placebo group and 7 (17.5%) among those treated with the drug.

The authors conclude that the prophylactic effect of the drug was slight, and that it could not be recommended for the prophylaxis of smallpox in view of the severity of its side-effects.

TREATMENT OF SMALLPOX

The use of M and B 7714 in the treatment of smallpox was investigated by Rao, McFadzean and Kamalakshi (1966a). The protocol used by these workers has already been described in the section of the treatment of smallpox with methisazone (p. 78). The drug was given by mouth as tablets in a dose of 1 g every 6 or 8 hr for 5 or 10 days, or as a suspension in a dose of 1.5 g or 2 g every 6 hr for 10 days, or 6 g twice daily for 10 days. Children and infants received proportionately lower doses.

The total number of patients admitted to the trial was 1293; of these, 601 received a placebo and 692 were treated with the drug. The mortality rates in these two groups were 23.3% (140 deaths) and 22.4% (155 deaths), respectively. Marks of previous vaccination were seen on 478 patients; 218 of these received the placebo and 8 died (3.7%), and the remaining 260 received the drug, of whom 5 died (1.9%). The mortality rates among 815 patients who had not been previously vaccinated were 132 of 383 (34.5%) given placebo and 150 of 432 (34.7%) given the drug. The differences in the figures were not significant. Some slight differences in favour of the drug were seen in analyses of the mean number of febrile days and the time to scabbing, but the differences were not significant.

The authors concluded that there was some evidence that the drug had a beneficial effect although none of the differences attained significance.

TOXICITY OF M AND B 7714

The chronic toxicity of M and B 7714 was evaluated in rats and monkeys, which were given the compound by mouth in daily doses of 250 or 1000 mg/kg for 3 months. In rats there was no evidence of liver or kidney dysfunction, but there was some toxic suppression of bone marrow and testicular damage. The monkeys showed no kidney dysfunction, but there was slight liver damage and moderate testicular atrophy. In view of the long period over which the drug was administered the adverse effects noted were insufficient to contra-indicate its use in man.

In the prophylactic trial the drug was not well tolerated; 74% of those taking it vomited, and 52% refused to complete the course. A drug rash was seen in 14%, and two children had febrile convulsions and died, although the cause of death was not definitely attributed to the drug.

In the therapeutic trial side-effects were much less noticeable. Nausea and vomiting occurred in some patients, but did not generally affect the

completion of the course of treatment. Vomiting could be controlled in some cases by preliminary administration of prochlorperazine. Four patients developed allergic dermatitis, which responded to antihistamine treatment.

Jaundice developed in 18 patients; it usually appeared after 3–4 days of treatment and reached maximum intensity between 5 and 11 days. The serum bilirubin values increased over the same period and reached a maximum of 4.1–9.4 mg/100 ml after 5–8 days. Normal values were regained after 20 days, and no abnormalities were seen in other tests of liver function. It was considered that the drug interfered with the conjugation of bilirubin with glucuronic acid, resulting in decreased excretion.

4-METHYL-5-FORMYLTHIAZOLE THIOSEMICARBAZONE

Another line of development of the early work on the antiviral thiosemicarbazones culminated in the synthesis and evaluation of 4-methyl-5-formylthiazole thiosemicarbazone by Campaigne, Thompson and Van Werth (1959). The structure is shown in Fig. 18, from which it can be seen that the compound is closely related to M and B 7714. When the compound was fed to mice in the diet in a concentration of 0.01% it conferred marked protection against intranasal infection with vaccinia virus. The activity was abolished if the side-chain was in the 4-position, but no other information is available on the structure–activity relationships of the compound. The activity was confirmed by Bauer (1965b) who found that mice infected intracerebrally with vaccinia virus could be completely protected against death by doses of 5 mg/kg given subcutaneously twice daily for 4 days. In spite of the high activity of the compound it has not been developed further for trial in man.

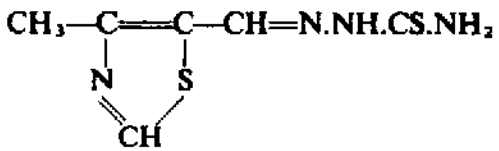


FIG. 18. Structural formula of 4-methyl-5-formylthiazole thiosemicarbazone.

BUTANE-2,3-DIONE OXIME THIOSEMICARBAZONE

Thompson *et al.* (1953b) found antiviral activity associated with aliphatic thiosemicarbazones, in particular butane-2,3-dione oxime thiosemicarbazone (Fig. 19, R = H). This observation was of considerable

interest, since it had previously been considered that an aromatic nucleus was essential for antiviral activity. The compound was administered in the diet in a concentration of 0.1% to 35 mice infected intracerebrally with vaccinia virus; 28 animals survived the infection, compared with 6 of 35 controls infected similarly, but not treated with the compound. The corresponding methoxime (Fig. 19, R = CH_3) was also active. The structure–activity relationships resembled those of the isatin thiosemicarbazones to a certain extent, since activity was abolished when the sulphur was replaced by oxygen (butane-2,3-dione oxime semicarbazone) or substituted with a methyl group (butane-2,3-dione oxime 5-methylthiosemicarbazone).

The activity was confirmed by Bauer (1953, 1965c) in mice treated with the compound subcutaneously in repeated doses of 5 mg/kg. At higher dose levels the compound was toxic.

The compound has not been developed further, but is of interest in showing that a cyclic structure is not essential for antiviral activity.

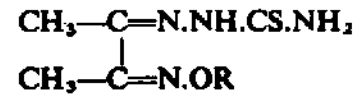


FIG. 19. Structural formula of butane-2,3-dione oxime thiosemicarbazone.

MISCELLANEOUS THIOSEMICARBAZONES

Lum and Smith (1957) found that the multiplication of the PR8 strain of influenza A and the Lee strain of influenza B in cultures of the chick chorioallantoic membrane could be inhibited by the thiosemicarbazones of *p*-hydroxybenzaldehyde, *p*-nitrobenzaldehyde and anisaldehyde in concentrations which were well below the maximum tolerated concentration. The compounds did not inactivate the virus on contact, and did not prevent adsorption and release; the action was therefore considered to be intracellular.

Gerzon (1965) found that 1-ethylpyrrolidine-2,3-dione thiosemicarbazone (Fig. 20, R = C_2H_5) would reduce the multiplication of the PR8 strain of virus in the lungs of mice infected by aerosol, and also confer protection against death, in comparison with untreated control animals infected in a similar manner. The β-hydroxyethyl analogue (Fig. 20, R = $CH_2OH.CH_2$) was slightly more active. No activity could be found in similar experiments with methisazone.

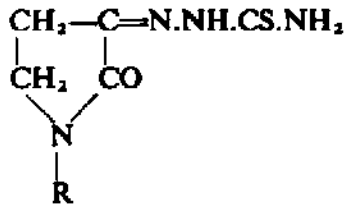


FIG. 20. Structural formula of 1-ethylpyrrolidine-2,3-dione thiosemicarbazone and derivatives.

Zak (1959) observed a slight reduction in the duration of the febrile period in 70 patients with influenza of unstated type treated with *p-iso*-propylbenzaldehyde thiosemicarbazone in a dose of 5 mg 3 times a day; the treatment was claimed to be effective but no information as to the significance of the effect was presented. No further work has appeared on the alleged antiviral activity of this compound.

MODE OF ACTION OF THE ANTIVIRAL THIOSEMICARBAZONES

The information at present available on the mode of action of the antiviral thiosemicarbazones has mostly been obtained with isatin 3-thiosemicarbazone. It is probably safe enough to assume that methisazone acts in the same way, but this assumption is not justified in the case of the aldehyde thiosemicarbazones, such as benzaldehyde thiosemicarbazone and M and B 7714, since the active species has not been established with certainty. The former compound can be cyclized to form 2-amino-5-phenylthiadiazole (Young and Eyre, 1901), and this was found to possess appreciable antiviral activity in mice infected with vaccinia virus (Bauer, 1965b). M and B 7714 is much more active by the oral than by the subcutaneous route, and it is possible that it is metabolized to an active derivative, although Caton *et al.* (1965) found that cyclization of the side-chain reduced or abolished the activity.

The isatin 3-thiosemicarbazones, being derived from ketones, are unable to undergo cyclization in the same way, but it has not been shown that the thiosemicarbazone is the active species, and it is quite possible that activity might be associated with metabolic modification of the side-chain.

Isatin 3-thiosemicarbazone does not inactivate virus directly, and does not prevent its attachment to cells (Sheffield *et al.*, 1960; Easterbrook, 1962). Its action is therefore intracellular. Easterbrook carried out a detailed study of the effect of the compound upon the multiplication of

vaccinia virus in KB cells. Concentrations of 10 μM or higher inhibited the production of infectious virus completely; below this concentration there was some proportionality between concentration of compound and yield of virus; a concentration of 0.1 μM was ineffective. The compound had no effect upon the production of virus antigen as determined by fluorescence microscopy, except in very high doses, and there was little effect upon the production of complement-fixing antigen. Cells pre-treated with the compound for 18 hr and then washed were able to support the multiplication of the virus in the normal way. This observation shows that the antiviral effect in tissue culture is not due to toxicity. and that the compound does not act by inducing interferon. It did not affect the incorporation of tritiated thymidine in the cytoplasm of infected cells, and therefore did not inhibit the synthesis of DNA.

The results thus indicated that isatin 3-thiosemicarbazone did not inhibit the production of the components of the virus, but prevented the subsequent formation of infectious virus. In order to establish the time relations of this process, cells were infected and grown in normal medium and at intervals portions of the cells were transferred to medium containing the compound; incubation was continued until the growth cycle was completed, and the amount of infective virus produced was determined by plaque counting. The results showed that the production of infective virus was inhibited when the cells were exposed to the compound after intervals ranging up to 6 hr, but with longer intervals an amount of virus was produced which was equal to that which had already developed in the cells by the time of addition.

In a converse experiment the infected cells were exposed to the compound at the beginning, and at intervals cells were transferred to medium not containing the compound and incubated further. The results showed that exposure for as little as 2 hr was sufficient to reduce the yield of infective virus, and the reduction was proportionately greater as the time of exposure was increased.

Similar experiments were carried out by Bach and Magee (1962), and Magee and Bach (1965), who used HeLa cells infected with vaccinia virus. The presence of isatin 3-thiosemicarbazone in the medium in a concentration of 3 μg/ml (13.6 μM) did not affect the uptake of tritiated thymidine into DNA by infected cells, or the uptake of valine-1-^{14}C into protein. The compound therefore did not inhibit the synthesis of virus DNA and protein, as found by Easterbrook.

Infected cells in which the multiplication of the virus was inhibited by isatin 3-thiosemicarbazone died nevertheless. It is evident that the early

stages of virus multiplication, which still proceed in the presence of the compound, cause sufficient disturbance to bring about the death of the cell.

Magee and Bach (1965) found that isatin 3-thiosemicarbazone and methisazone had little or no effect on the synthesis of virus DNA, and DNA made in the presence of these compounds could still be incorporated into mature virus particles. They concluded that it was unlikely that any direct interaction occurred between these compounds and DNA.

Appleyard, Hume and Westwood (1965) also carried out time of addition studies with isatin 3-thiosemicarbazone and found that the compound acted late in the cycle. However, when the compound was added to the cultures at the same time as the virus and removed after various intervals, the final yield of virus was reduced by 75% when the compound was removed after only 2 hr. This observation is not in agreement with the work of Bach and Magee, and it appears that the compound also has some effect in the early stages of the cycle. The effect upon the production of virus antigens was studied by immunodiffusion analysis of extracts of cells taken at intervals throughout the growth cycle. The compound had no effect upon the production of those antigens which appear during the first 4 hr of the growth cycle, but antigens which normally appear after this time were suppressed. They made the additional observation that the virus varied in its susceptibility to isatin 3-thiosemicarbazone according to the type of cell in which it was growing. Thus, a 90% reduction in virus yield was produced by a concentration of 0.1 μg/ml when the virus was growing in HeLa cells, but 0.6 μg/ml was required in primary human embryo kidney cells and 10 μg/ml in L cells. The reason for this is unknown. It is possible that the compound penetrates different cells to different extents, or may vary in the extent of its destruction by metabolic processes, but the explanation may equally well be in differences in the protein synthesizing mechanisms of the host at the molecular level.

Appleyard *et al.* (1965) also made a limited study of the mechanism of action of M and B 7714. It prevented the appearance of late virus antigens and did not affect the synthesis of virus DNA, and thus generally resembled isatin 3-thiosemicarbazone in its action.

They also observed that the antiviral effect of isatin 3-thiosemicarbazone could be reversed by actinomycin D. In a typical experiment, HeLa cells were exposed to actinomycin D in a concentration which was too low to have any inhibitory effect upon virus multiplication. After 3 hr the compound was removed and cultures were treated with normal medium. Three hours later they were infected with rabbitpox virus and treated with

isatin 3-thiosemicarbazone in various concentrations. The yield of virus after 24 hr was determined by plaque titration and compared with that obtained in cultures treated with isatin 3-thiosemicarbazone without preliminary exposure to actinomycin D. The results showed that the inhibition of virus yield produced by isatin 3-thiosemicarbazone was reversed by actinomycin D; the greatest reversal was obtained with a concentration of 0.4 μg/ml, and at higher concentrations actinomycin D itself caused inhibition of virus multiplication. A similar reversal was obtained with *p*-fluorophenylalanine in a concentration of 400 μg/ml. In time of addition studies it was found that the process sensitive to *p*-fluorophenylalanine began within 30 min of the addition of isatin 3-thiosemicarbazone. The authors conclude that DNA-dependent RNA synthesis (transcription of DNA) is an essential process in the inhibition produced by isatin 3-thiosemicarbazone. The newly formed RNA would presumably direct the synthesis of a protein which itself might be the actual antiviral agent.

Interferon comes to mind in this connexion, but Lieberman, Pollikoff and Pascale (1966) could not detect any interferon in the brains of mice infected intracerebrally with vaccinia virus and protected by a subcutaneous injection of 1-ethylisatin 3-thiosemicarbazone.

Evidence for the production of an antiviral substance by a thiosemicarbazone was obtained by Squires and McFadzean (1966). Mice were infected intracerebrally with vaccinia virus and protected by oral treatment with M and B 7714. After 96 hr the brains were removed and made into a 10% suspension which was freed from virus by centrifugation. The supernatant fluid was injected intradermally into rabbits, and 24 hr later the same sites were infected with vaccinia virus by scarification. No local lesions developed, whereas lesions developed in the usual way in rabbits treated with extracts of normal mouse brain, and of the brains of mice infected with vaccinia but not treated with M and B 7714. These observations indicate that an interfering factor is produced by M and B 7714 acting in conjunction with an infection with vaccinia virus.

Results obtained by conventional virological methods have thus shown that the thiosemicarbazones act late in the virus growth cycle and inhibit the formation of certain proteins. This conclusion is supported by the work of Easterbrook (1962), who found immature or abnormal virus particles in cells infected with vaccinia virus and treated with isatin 3-thiosemicarbazone. It is also possible that the thiosemicarbazones have an additional action which is exercised during the early part of the growth cycle.

The precise mechanism of action has been elucidated by Woodson and Joklik (1965) in an investigation carried out by the sophisticated methods of molecular biology.

When the virus DNA has replicated the progeny DNA molecules are coated with protein in the process of formation of infective virus particles. When the molecules have been coated they are no longer susceptible to the action of DNAse. In order to follow this process the DNA is labelled with ^{14}C; treatment with DNAse will release the label at first, but the amount released becomes successively lower as the DNA becomes coated and inaccessible to the enzyme. By this means Woodson and Joklik showed that isatin 3-thiosemicarbazone in a concentration of 15 μM reduced the amount of DNA coated by at least 70%. The amount of DNA incorporated into inactive virus particles was reduced by about 90%. These findings suggested that the compound inhibited the synthesis of virus protein, and were thus in agreement with the inhibition of late virus antigens found by Appleyard *et al.* (1965).

In further work the synthesis of virus protein was followed by measuring the uptake of labelled amino-acids. In the presence of isatin 3-thiosemicarbazone in a concentration of 15 μM the synthesis of virus protein took place at a normal rate for the first 3 hr of the growth cycle, but after this time a marked reduction set in. After 4 hr the amount of protein synthesized was inhibited by 80% and after 6 hr the inhibition was 90%.

Inhibition of protein synthesis could be due to inhibition of the synthesis of messenger RNA or to inhibition of the translation process, and these two possibilities were investigated next.

The messenger RNA of vaccinia RNA is formed in the cytoplasm, whereas RNA transcribed from the host cell DNA is found at first in the nucleus. If uridine labelled with ^{14}C is supplied to the infected cells, and the cells are separated into nuclear and cytoplasmic fractions 30 min later, any label found in the latter fraction represents virus messenger RNA, and it can be determined quantitatively. By using this method it was found that the synthesis of virus messenger RNA was unaffected by 15 μM isatin 3-thiosemicarbazone.

The messenger RNA becomes attached to ribosomes to form accumulations known as polyribosomes, which translate the code and form the polypeptides coded in the virus genome. The polyribosomes can be separated and quantitated by density gradient centrifugation. In the presence of isatin 3-thiosemicarbazone the virus messenger RNA forms polyribosomes in the normal manner for the first 4 hr of the multiplication cycle of the virus, but after this time the peak representing polyribosomes

in the density gradient fractions falls off and the peak representing single ribosomes increases in size. The polyribosomes formed after 4 hr thus become unstable in the presence of the compound, and the messenger RNA can be detected in uncombined form.

The sedimentation coefficient of the messenger RNA, which affords a measure of its size, was also determined. The RNA which is formed in the presence of isatin 3-thiosemicarbazone has the normal sedimentation constant of 16S, but within a few minutes the value falls to 8S as the result of some kind of degradation. The messenger RNA then cannot exert its function, and the synthesis of the proteins which it represents comes to an end. This is in accordance with the suppression of the formation of late proteins which was observed in immunodiffusion experiments.

The mechanism by which isatin 3-thiosemicarbazone affects the messenger RNA is not yet known, but it may be of significance that the activity of the compound comes on at a time when virus progeny DNA is accumulating in the cytoplasm and is being transcribed. In view of the results with actinomycin D, it is possible that the compound affects the synthesis of a virus protein at the transcription or translation level and converts it from its normal function to the inactivation of virus messenger RNA.

How isatin 3-thiosemicarbazone exerts its action is not known in chemical terms. The thiosemicarbazones form metal chelates and might have biological activity for this reason. Thompson *et al.* (1953b) attempted to reverse the antiviral action of isatin 3-thiosemicarbazone and certain aliphatic thiosemicarbazones with metal salts. Mice were infected intracerebrally with vaccinia virus and protected against death by administering the appropriate thiosemicarbazone in the diet. No reversal of the protection effect occurred when the diet contained also copper chloride (0.05%), cobalt nitrate (0.05%), zinc chloride (0.1%), manganese chloride (0.5–1.0%) or sodium molybdate (0.015%). Other chelating agents which were not thiosemicarbazones had no antiviral effect. It was concluded that there was no evidence that chelation was involved in the mechanism of antiviral activity of the thiosemicarbazones. No further work has been reported on this subject, and it is very difficult to devise an experiment to demonstrate unequivocally that the antiviral effect of thiosemicarbazones is due to chelation at the site of action.

RESISTANCE TO THIOSEMICARBAZONES

Pox viruses will become resistant to thiosemicarbazones when passaged in the presence of increasing amounts of the compound. This type of

resistance is similar to that found in bacteria. There is, however, another type of resistance, which appears so far to be confined to the pox viruses, since the sensitivity of the virus to a given compound depends also upon the cell line in which it is growing. In the pox virus–thiosemicarbazone interrelationship there is thus resistance by adaptation, and cell-dependent resistance.

RESISTANCE BY ADAPTATION

The first observations were made by Thompson *et al.* (1953b). Mice were infected intracerebrally with the Williamsport strain of vaccinia virus and given isatin 3-thiosemicarbazone intraperitoneally in a dose of 5 mg on the day of infection and the two following days. After 5 days the brains were removed and pooled; the material was used for a subsequent passage under the same conditions, and was also titrated on the skin of rabbits. The titre fell from 5.1 to 2.8 log units after 4 passages, whereas in a control series in which the animals were untreated the titre rose from 5.8 to 7.3 log units. In a similar experiment with doses of 2.5 mg the titre fell from 5.5 to 1.4 log units over 7 passages. There was thus no evidence of the development of resistance.

Different results were obtained by Appleyard and Way (1966). The Utrecht strain of rabbitpox virus was passaged intranasally in mice which were treated with 0.5 or 1.0 mg of methisazone daily. Plaque-reduction tests were carried out on monolayers of HeLa cells against isatin 3-thiosemicarbazone with virus obtained from mouse lungs at successive passage levels. The results are shown in Fig. 21. It is evident that the dose–response curve shifts towards higher dose levels during three successive passages in treated mice, and the virus is thus developing resistance. The virus had also lost its sensitivity to methisazone when tested in mice.

The development of resistance was also demonstrated in tissue culture. Rabbitpox virus was carried through serial passages in cultures of HeLa cells containing isatin 3-thiosemicarbazone in concentrations rising from 0.05 to 20 μg/ml. Successive passage levels of the virus were tested against isatin 3-thiosemicarbazone in plaque-reduction tests. The dose–response curves thus obtained moved towards higher concentrations of the compound. The original virus was almost completely inhibited by 0.1 μg/ml, but after 15 passages a concentration of 10 μg/ml inhibited plaque production by only 50%. The virus had therefore become more than 100 times less sensitive. The same effect was noted when the total virus yield of the cultures was determined in the presence of varying concentrations

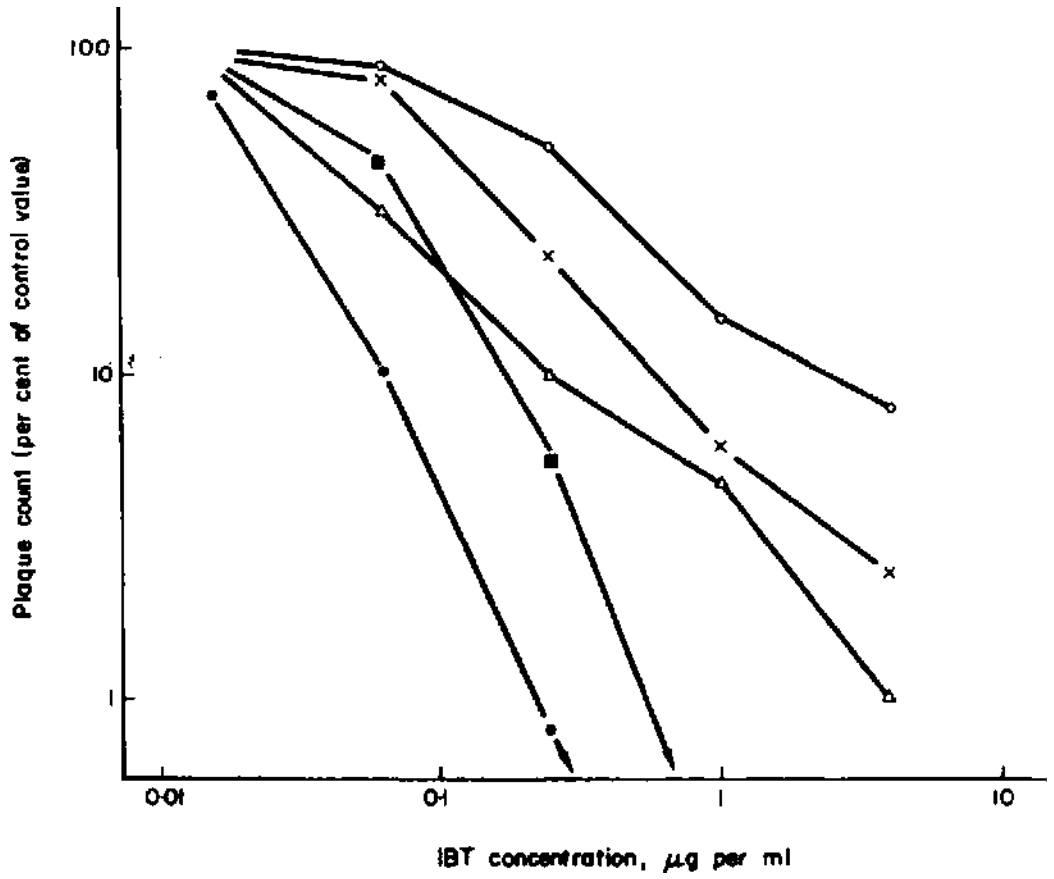


FIG. 21. Development of resistance to isatin 3-thiosemicarbazone in rabbitpox virus. Dose–response lines of culture-adapted virus (—●—), virus passed 5 times in normal mice (—■—), and passed twice in normal mice and once (—△—), twice (— × —) and three times (—○—) in mice treated with methisazone. The development of resistance is shown by the displacement of the dose–response lines to higher concentrations of compound (Appleyard and Way, 1966).

of the compound (Fig. 22). The yield of the original virus was almost completely inhibited by 1 μg/ml, whereas concentrations of 1–10 μg/ml had little effect upon the yield of adapted virus.

Virus which had become resistant to isatin 3-thiosemicarbazone was completely resistant to methisazone. It was also resistant to the monocyclic thiosemicarbazone M and B 7714, but to a somewhat lesser extent. This cross-resistance indicates that the compounds have a common mechanism of action. There was no cross-resistance with *p*-fluorophenylalanine or 5-bromo-2′-deoxyuridine.

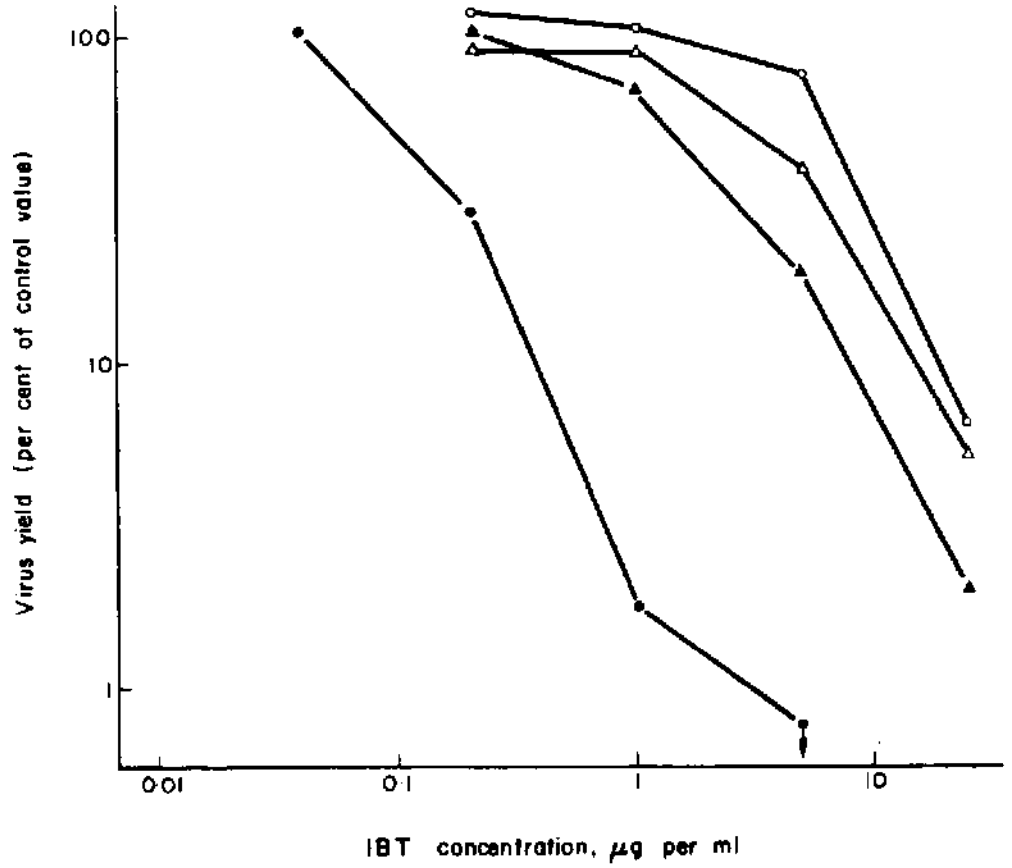


FIG. 22. Development of resistance to isatin 3-thiosemicarbazone in rabbitpox virus. Dose–response lines of normal virus in HeLa (—●--) and L (—▲—) cells, and of resistant virus in HeLa (—○—) and L (—△—) cells. The extent of the drug resistance is shown by the displacement of the dose–response lines to higher concentrations of compound. Normal virus is less susceptible in L cells than in HeLa cells (Appleyard and Way, 1966).

CELL-DEPENDENT RESISTANCE

Appleyard, Hume and Westwood (1965) found that the sensitivity of rabbitpox virus to isatin 3-thiosemicarbazone was lower in RK 13 cells and L cells than in HeLa cells. The yield of virus was reduced by about 2 log units by concentrations of 0.5–2 μg/ml, but a concentration of 32 μg/ml was required to effect this degree of reduction in the other two cell lines. They considered that the compound was 100 times less effective in RK 13 and L cells. There was no evidence that the cell lines differed in their permeability to the compound, and the toxicity of the compound was about the same in all three cases. Cell-dependent resistance does not appear to be known for other systems; it is of potential importance in clinical practice, since a chemotherapeutic effect demonstrable in animals

may not occur to the same extent in man, and if it does, the compound may not be equally effective in all tissues. Appleyard and Way (1966) found that rabbitpox virus adapted to isatin 3-thiosemicarbazone gave a dose–response curve to this compound in L cells which was similar to that of unadapted virus in L cells. This suggests that there may be a close relationship between the two kinds of resistance, probably at the molecular level.

ISATIN 3-DIALKYLTHIOSEMICARBAZONES

Substitution of a methyl group in the 4′-position of the side-chain of isatin 3-thiosemicarbazone greatly reduces the activity against vaccinia, and substitution of 2 groups completely abolishes it (Bauer and Sadler, 1960a). It was subsequently found that the dimethyl derivative is active against ectromelia (Bauer and Sadler, 1961). Mice infected intracerebrally with 10 LD_{50} of the Sandom strain of ectromelia virus were completely protected by treatment with isatin 3-(4′,4′-dimethyl)thiosemicarbazone in a dose of 25 mg/kg given subcutaneously twice daily for 5 days. When the virus dose was increased to 100 LD_{50} protection was still observed, in that 50% of the mice survived. The compound in the same dose also conferred protection in mice infected with 10–10,000 LD_{50} of virus injected intraperitoneally. The 1-methyl derivative was considerably more active. In a dose of 50 mg/kg twice daily for 5 days it completely protected mice infected intraperitoneally with 1000 LD_{50} of virus, and some protection was observed when the same dose of virus was given intracerebrally.

In further work (Bauer, 1963) the structure–activity relationships were investigated. Substitution in the aromatic ring caused some loss of activity, but not nearly so much as in the case of isatin 3-thiosemicarbazone and vaccinia virus. Activity against ectromelia was reduced by increasing the chain length of the substituents in the 4′-position (ethyl, propyl, butyl) and in the 1-position. The structure–activity relationships thus differ greatly from those observed with vaccinia, and must be a reflection of differences in components of the respective viruses at the molecular level. High activity was still observed when the terminal part of the side-chain was cyclic, as in isatin 3-(4′,4′-tetramethylene)thiosemicarbazone and its pentamethylene analogue.

The antiviral spectrum of the isatin 3-(4′,4′-dialkyl)thiosemicarbazones was extended by Pearson and Zimmerman (1969), who found that the dibutyl derivative inhibited the multiplication of all 3 types of poliomyelitis virus in HeLa cells. The compound inhibited the synthesis of

virus RNA, the formation of the replication complex and the activity of the virus-specific RNA polymerase. The inhibition was thus due to a specific effect on the synthesis of virus RNA, and not to an unspecific toxic effect on the cells.

The isatin 3-(4′,4′-dialkyl)thiosemicarbazones have not been used in man.

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CHAPTER 2

2-(α-HYDROXYBENZYL)BENZIMIDAZOLE AND RELATED COMPOUNDS

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INTRODUCTION AND HISTORY

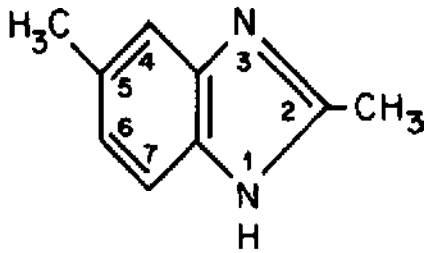
EXTENSIVE studies of the virus-inhibitory activity of benzimidazole derivatives started in 1952 from the idea that derivatives of this bicyclic compound might be found which would be capable of inhibiting virus multiplication selectively (Tamm *et al.*, 1952). The underlying assumption was that the biological specificity of viruses is determined by or associated with specific nucleic acids and proteins. Inhibition of virus multiplication without interfering with the synthesis or function of host cell nucleic acids and proteins seemed possible, provided that inhibitors which were sufficiently selective could be found. There was evidence in 1952 that well-known analogs of precursors of nucleic acids and proteins had a non-selective effect on virus multiplication (cf. reviews by Tamm, 1958, 1959). This left open the possibility that selective inhibition of virus nucleic acid synthesis might be obtained with synthetic compounds or biological substances, which would act on a mechanism controlling nucleic acid biosynthesis. In 1952 a feasible experimental approach seemed to be the study of structural analogs of vitamins known to play a role in nucleic acid biosynthesis. Vitamin B_{12} is one such vitamin. It contains a benzimidazole nucleoside moiety, 5,6-dimethyl-1-α-D-ribofuranosylbenzimidazole. In studies with benzimidazole derivatives so far carried out, no evidence has been obtained linking the virus-inhibiting activity of the derivatives with the function of vitamin B_{12} in nucleic acid biosynthesis. However, a selective inhibitor of virus nucleic acid synthesis has been found among

benzimidazole derivatives. 2-(α-Hydroxybenzyl)benzimidazole (HBB) inhibits the replication of the virus RNA of numerous picornaviruses (Eggers and Tamm, 1962a, 1963a), but has no effects on cell RNA synthesis or on the replication of the nucleic acid of other viruses. Numerous derivatives of HBB also possess selective inhibitory action on the multiplication of picornaviruses. Besides HBB and its derivatives, there are several other groups of biologically active benzimidazoles, each characterized by special structural features and possessing striking and different biological activities.

The conclusion was reached early in the course of studies of structure–activity relationships that the virus-inhibiting activity and toxicity of benzimidazole derivatives can vary independently (Tamm, 1955, 1956a, b). For a number of years, however, all the results indicated that at virus-inhibitory concentrations, benzimidazole derivatives had significant effects on host cells as judged by morphological or metabolic criteria (Tamm, 1958). Yet the fact that benzimidazole derivatives did vary in relative selectivity indicated that appropriate structural modification might ultimately give highly selective virus-inhibitory compounds.

ALKYL DERIVATIVES OF BENZIMIDAZOLE

The first benzimidazole derivative examined was 2,5-dimethylbenzimidazole (Fig. 1) (Tamm *et al.*, 1952). This compound was neither highly active nor selective as an inhibitor of influenza B virus multiplication in the chorioallantoic membrane *in vitro*. However, its reversible inhibitory effect on cell processes permitted the first demonstration that a biosynthetic product of the host is implicated in virus-induced interference of virus multiplication (Tyrrell and Tamm, 1955). The significant finding is



2,5-Dimethyl-benzimidazole

FIG. 1. The structure of 2,5-dimethylbenzimidazole—a non-selective inhibitor of biosynthesis.

that 2,5-dimethylbenzimidazole prevents the induction of interference by heat-killed influenza virus. While killed virus is able to induce a state of interference in untreated cells without itself multiplying, it is not able to do so in cells in which biosynthesis is inhibited by 2,5-dimethylbenzimidazole.

Studies of a series of alkyl derivatives of benzimidazole have shown that more extensive substitution in either the benzyl or the imidazole ring results in a marked increase in inhibitory activity on influenza B virus multiplication (Tamm *et al.*, 1953). In some instances, toxicity varies in parallel with virus-inhibitory activity, but in others, it varies independently. For example, the 2-ethyl-5-methyl derivative of benzimidazole is not only much more active than the 2,5-dimethyl compound, but it is also less toxic at equivalent virus-inhibitory concentrations. Further lengthening of the alkyl side chain at carbon 2 by one or two alkyl residues (giving propyl and butyl derivatives) does not increase activity (Tamm *et al.*, 1953). However, the 2-amyl-5-methyl derivative and the 2-heptyl-5-methyl derivative do show further increases in activity (Tamm *et al.*, 1961).

N-GLYCOSIDES OF HALOGENATED BENZIMIDAZOLES

Studies with derivatives containing halogen substituents have confirmed the potentiating effect on inhibitory activity of introduction of multiple substituents in the benzo moiety (Tamm *et al.*, 1954; Tamm, 1954; Tamm *et al.*, 1956). Furthermore, conversion of halogenated benzimidazoles to the corresponding β-linked *N*-ribosides increases both the influenza virus-inhibitory activity and the selectivity of compounds. The derivatives which have been studied most extensively are 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB) (Fig. 2) and the corresponding trichloro derivative (TRB). The β-linked ribofuranosides of halogenated benzimidazoles are highly active inhibitors of cell RNA synthesis (Tamm, 1958; Tamm *et al.*, 1960; Sirlin and Jacob, 1964; Bucknall, 1967). These derivatives have played a significant part in studies of the requirements and mechanism of virus multiplication (Tamm and Tyrrell, 1954; Tamm, 1958; Tamm *et al.*, 1960; Ikegami *et al.*, 1960; Bucknall, 1967). They have also been useful tools in investigations of the role of RNA in protein synthesis (Allfrey *et al.*, 1957; Allfrey and Mirsky, 1957) and of the different species of cell RNA (Sirlin and Loening, 1968). DRB appears to act by interfering with the incorporation of adenosine into RNA (Tamm *et al.*, 1960). Adenosine, but not guanosine, is capable of partially blocking the inhibitory effects of DRB.

The high inhibitory activity of *N*-glycosides of halogenated benzimidazoles on influenza virus multiplication depends both on the halogen and carbohydrate substituents. The type of halogen substituent is not especially important, but the activity increases markedly as the number of halogen atoms in the benzo moiety is increased from one to four (Tamm *et al.*, 1956). For highest inhibitory activity the carbohydrate must not only be ribose but specifically ribofuranose in the β-linkage is required.

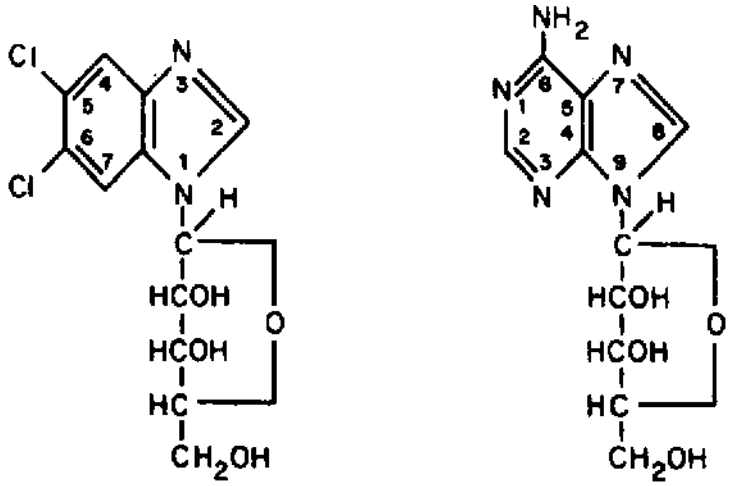


FIG. 2. The structures of 5,6-dichloro-1-β-D-ribofuranosylbenzimidazole (DRB)—an inhibitor of RNA synthesis; and of adenosine—a precursor of RNA.

The first evidence that synthesis of RNA is a necessary step in the reproduction of DNA-containing animal viruses was obtained with the aid of DRB. This compound, which inhibits RNA synthesis, not only inhibits the RNA-containing influenza virus, but is also an effective inhibitor of the multiplication of the DNA-containing vaccinia (Tamm and Overman, 1957) and adenoviruses (Tamm *et al.*, 1960). It is now well known that the synthesis of virus-specific messenger RNA is a critical step in the multiplication of DNA viruses.

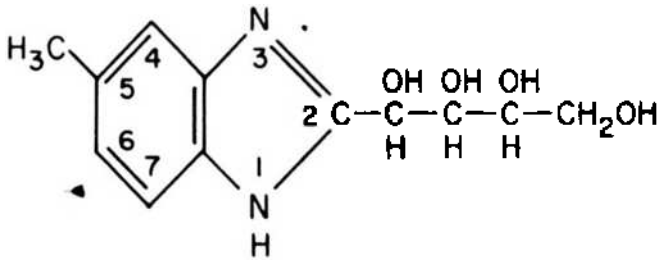
The relatively low sensitivity of poliovirus multiplication to inhibition by DRB indicates (Tamm and Nemes, 1957; Tamm *et al.*, 1960) that poliovirus multiplies independently of some DRB-sensitive RNA synthesis in the infected cell. Replication of poliovirus is not significantly inhibited by actinomycin D (Reich *et al.*, 1962; Shatkin, 1962), whereas that of influenza virus is markedly inhibited (Barry, 1964). Taken together, this

is strong evidence that in the biosynthesis of influenza virus there is a requirement for a species of RNA which is not required in poliovirus replication. This as yet unidentified species is made early during the latent period in the growth cycle of influenza virus (Tamm and Tyrrell, 1954).

Besides being useful in studies of the metabolic requirements of virus synthesis, β-linked ribofuranosides of halogenated benzimidazoles have provided additional evidence that virus-inhibitory activity and toxicity can vary independently.

2-(POLYHYDROXYALKYL)BENZIMIDAZOLES

Attempts to obtain more active and selective inhibitors through substitution of large groups at position 2 in the imidazole ring have given surprising results of considerable interest. 5-Methyl-2-D-ribobenzimidazole (MRB) (Fig. 3) and certain other 2-(polyhydroxyalkyl)benzimidazoles



5-Methyl-2-D-ribobenzimidazole
(MRB)

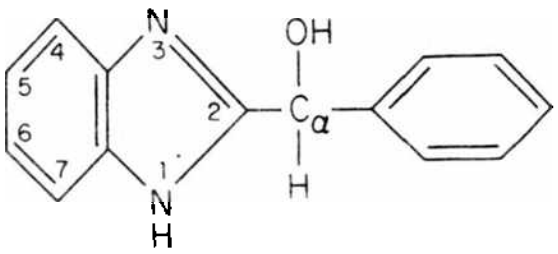
FIG. 3. The structure of 5-methyl-2-D-ribobenzimidazole (MRB)—a selective enhancer of influenza virus multiplication.

enhance rather than inhibit the yield of certain strains of influenza A or B virus in the chorioallantoic membrane of embryonated chicken eggs (Tamm, 1956c). MRB restores the ability of tissues from older embryonated eggs to produce maximal amounts of virus. MRB was the first benzimidazole derivative to show virus-specific effects. The presence of hydroxyl groups on the side chain appears to be essential for enhancing activity.

2-(α-HYDROXYBENZYL)BENZIMIDAZOLE (HBB)

Examination of additional derivatives containing large substituents at position 2 in the imidazole ring of the benzimidazole molecule led to the

discovery of the first selective inhibitor of picornavirus replication. It was demonstrated more than ten years ago that 2-(α-hydroxybenzyl)-benzimidazole (HBB) (Fig. 4) inhibits the multiplication and cytopathic



2-(α-Hydroxybenzyl)-
benzimidazole
(HBB)

FIG. 4. The structure of 2-(α-hydroxybenzyl)benzimidazole (HBB)—a selective inhibitor of picornavirus multiplication.

effects of poliovirus in primary cultures of monkey kidney cells (Hollinshead and Smith, 1958; Tamm and Nemes, 1959), and that it has a slight protective effect against experimental poliovirus infection in the mouse (Hollinshead and Smith, 1958). The selective action of HBB on the multiplication of picornaviruses was established when it was shown that this compound has no effect on the multiplication of viruses belonging to other major groups and that in fact not all picornaviruses are sensitive (Tamm and Nemes, 1959; Tamm *et al.*, 1961; Eggers and Tamm, 1961a, b). This is in itself evidence that HBB does not act by inhibiting some vital metabolic activity of host cells; if it did, the multiplication of viruses in other major groups would be expected to be affected. Furthermore, substantial direct evidence has been obtained that at concentrations sufficient to inhibit markedly the multiplication of sensitive picornaviruses, HBB has no effects on the metabolic activity, morphological appearance, or division of uninfected cells (Eggers and Tamm, 1961a, 1962a). The virus-specific action of HBB is directed against synthesis of the RNA of the HBB-sensitive picornaviruses (Eggers and Tamm, 1962a, 1963a). There is considerable evidence that inhibition of formation of virus proteins, virus particles and of virus cytopathic effects in treated cells are secondary phenomena (Eggers and Tamm, 1962a; Halperen *et al.*, 1964a, b; Bablanian *et al.*, 1966; Skinner *et al.*, 1968). The structural requirements for selective virus-inhibiting activity of HBB have been defined (Tamm

et al., 1961; Tamm *et al.*, 1969; O'Sullivan, 1965) and a number of derivatives have been synthesized that are more active and selective than HBB in inhibiting picornavirus replication.

VIRUS-INHIBITORY SPECTRUM

The virus-inhibitory action of HBB has been evaluated on the basis of inhibition of virus yield or reduction of virus cytopathic effects. The relationship between inhibition of yield of poliovirus 2 and of virus-induced cell damage is illustrated in the upper part of Fig. 5 (Tamm *et al.*, 1961). The curves describing the relationship between concentration of compound and inhibition of virus yield or reduction in virus-induced cell damage are approximately parallel. However, the concentration required to inhibit virus-induced cell damage by 75% is 1.55 times higher than that required to reduce virus yield by 75%. The concentration (56 μM), which inhibits virus-induced cell damage by 75%, reduces the yield of virus by 95%. To cause a marked reduction in virus cytopathic effects, it is clearly necessary to inhibit virus multiplication to a very great extent. In agreement with these results is the finding, obtained with guanidine and poliovirus, that less than maximal virus multiplication is sufficient to lead to maximal virus cytopathic effects (Bablanian *et al.*, 1965b). Demonstration of inhibition of virus cytopathic effects by HBB or guanidine can be taken as strong presumptive evidence of significant inhibition of virus multiplication. The concentration–effect relationships recorded in Fig. 5 are strictly valid only for the virus inoculum (500 $TCID_{50}$) and incubation time (48 hr) used.

HBB at a concentration of 493 μM causes slight morphological changes in 48 hr in monkey kidney cells in culture, while 219 μM HBB is non-toxic (Fig. 5, upper part). Some compound-induced morphological changes are observed after 3–7 days' incubation with 329 μM HBB, while 219 μM HBB causes no apparent cytotoxicity even after 7 days (Fig. 5, lower part).

HUMAN ENTEROVIRUSES

The ability of HBB to inhibit viral multiplication or cytopathic effects has been investigated with viruses belonging to seven major groups. The outstanding finding is that HBB selectively inhibits many, but not all, picornaviruses. Table 1 shows that 219 μM HBB completely inhibits the multiplication and cytopathic effects of representative coxsackie B and echoviruses in monkey kidney cells, but has no reproducible or significant

effects on three myxoviruses, vaccinia virus, and echovirus 22 (Eggers and Tamm, 1961a).

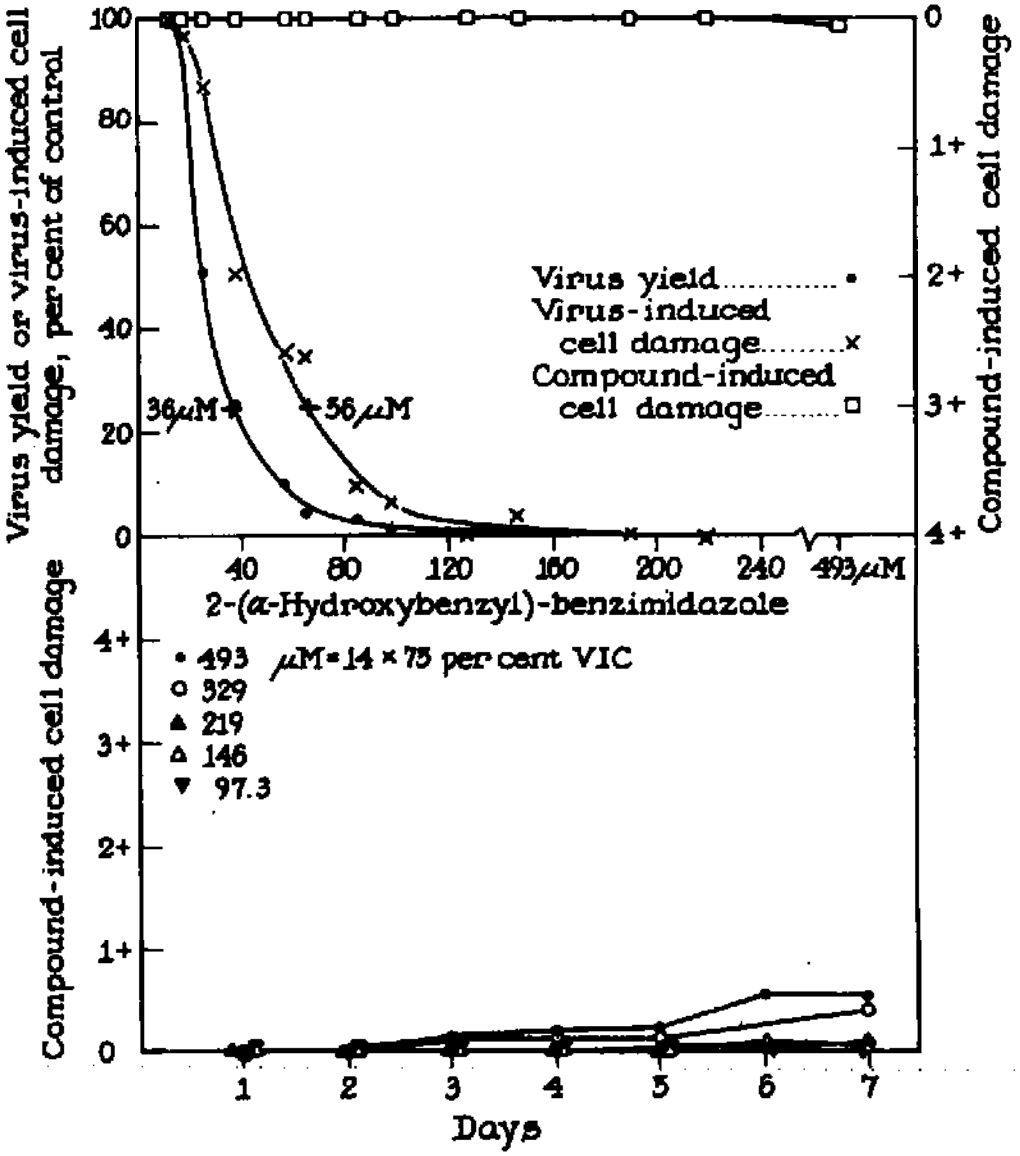


FIG. 5. Relationship between concentration of 2-(α-hydroxybenzyl)benzimidazole (HBB) and (a) inhibition of multiplication and cytopathic effects of poliovirus 2 (upper part), and (b) toxicity of the compound in monkey kidney cells (lower part).

Top part of Fig. 5: Primary monolayer cultures of rhesus monkey kidney cells were inoculated with 500 $TCID_{50}$ of poliovirus 2 per 2.5×10^5 cells. The medium contained varying concentrations of HBB. The cultures were incubated for 48 hr at which time the extent of virus cytopathic effects and the virus yields in control and treated cultures were determined (From Tamm *et al.*, 1961.)

Bottom part of Fig. 5: Uninfected cultures were incubated with HBB at a series of concentrations and observed daily for a period of 7 days. Extent of microscopic damage was noted. (From Tamm and Nemes, 1959; Tamm *et al.*, 1961.)

TABLE 1. INHIBITION OF VIRUS MULTIPLICATION AND CYTOPATHIC EFFECTS BY 2-(α-HYDROXYBENZYL)BENZIMIDAZOLE (HBB) IN PRIMARY CULTURES OF RHESUS MONKEY KIDNEY CELLS

Virus	Inoculum	Incubation[c]	Virus yield[a]		Cell damage[b]	
			Control	HBB 219 μM	Control	HBB 219 μM
	$TCID_{50}$	days				
Coxsackie B4 (Powers)	320	3	>8.2	<1.8	100	0
Echo 6 (D'Amori)	320	4	8.7	<1.5	94	0
Echo 7 (Wallace)	25	3	8.6	<1.5	100	0
Echo 9 (Hill)	100	7	7.3	<1.5	63	0
Croup-associated (Greer)	56	6	6.4	6.3	78	47
Hemadsorption 1 (C-243)	56	6	7.2	6.9	88	81
Mumps (Utz)	200	7	5.8	5.1	41	41
Vaccinia	56	6	6.9	6.6	89	94
Echo 22 (Morrison)	10,000	5	6.9	6.6	71	83
Echo 22 (R. Host)	3200	4	5.3	5.8	25	31

[a] Expressed as log $TCID_{50}$ per ml.
[b] Expressed as per cent cells affected.
[c] Protein-free Eagle's minimum essential medium.
From Eggers and Tamm, 1961a.

The results of the largest comparative study are summarized in Table 2. In this study inhibition of virus cytopathic effects by HBB was determined. With most viruses, primary cultures of rhesus monkey kidney cells and protein free Eagle's minimum essential medium were used (Eggers and Tamm, 1961a, b; Tamm and Eggers, 1962). With some viruses, other cell types such as human embryonic lung cells were used, as viruses differ in their host requirements. In most experiments 30 to 500 $TCID_{50}$ of virus was inoculated per culture tube, but in some tests larger inocula were used. Infected cultures were incubated in the presence or absence of HBB. HBB was usually used at three concentrations, i.e., 98, 219, and 493 μM. The cultures were incubated at 36°C, and examined daily or every other day. The final reading was made 7 or 8 days after inoculation. Virus-induced cell damage was evaluated in terms of the percentage of cells affected.

TABLE 2. INHIBITION OF VIRUS CYTOPATHIC EFFECTS BY HBB[a]

A Complete inhibition by 98 μM HBB	B Complete inhibition by 219 μM HBB, but not by 98 μM HBB	C Incomplete inhibition by 219 or 493 μM HBB	D No inhibition by 219 or 493 μM HBB
Coxsackie B4 (Powers)	Coxsackie A9 (Woods)	Coxsackie A21 (Coe)	Coxsackie A7 (AB IV Habel)
Echo 4 (Pesascek)	Coxsackie B1 (P.O. Dalldorf)	Echo 20 (JV-1)	Coxsackie A11 (D 52148)
Echo 6 (D'Amori)	Coxsackie B2 (Ohio 1)	Polio 1 (Mahoney)	Coxsackie A13 (D 5359)
Echo 9 (Hill)	Coxsackie B3 (Nancy)	Polio 2 (MEF1)	Coxsackie A16 (D 52109)
Echo 13 (11-4-1D)	Coxsackie B5 (Faulkner)	Polio 3 (Saukett)	Coxsackie A18 (D 52112)
Echo 14 (Tow)	Coxsackie B6 (1-51-21)		Echo 22 (Harris)
Echo 15 (Charleston 96-51)	Echo 1 (Farouk)		Echo 23 (Williamson)
Echo 17 (CHHE-29)	Echo 2 (Cornelis)		Rhino 1A (2060)
Echo 18 (Metcalf)	Echo 3 (Morrisey)		Rhino 1B (B632)
Echo 24 (de Camp)	Echo 5 (Noyce)		Rhino 2 (HGP)
Echo 26 (11-3-6)	Echo 7 (Wallace)		Reo 1 (Lang)
	Echo 8 (Bryson)		Reo 2 (Jones)
	Echo 11 (Gregory)		Reo 3 (Dearing)
	Echo 12 (Travis 2-85)		Arbo B (West Nile)
	Echo 16 (Harrington)		Arbo C (Marituba; Oriboca)
	Echo 19 (Burke)		Influenza B (1760)
	Echo 21 (Farina)		Parainfluenza 2 (Greer)
	Echo 25 (JV-4)		Parainfluenza 3 (C-243)
	Echo 27 (1-36-4)		Mumps (Utz)
			Adeno 2 (Ind-2)
			Adeno 3 (JF)
			Adeno 4 (RN)
			Herpes simplex (RE)
			Vaccinia

[a] Generally, primary cultures of rhesus monkey kidney cells were used, except that coxsackievirus types 11, 13, and 18 were studied in ERK and HEL cells; rhinovirus types 1A, 1B and coxsackievirus A21 in HEL cells; rhinovirus types 1A and 2 were also studied in monkey kidney cells; herpes virus was examined in HeLa cells. The following media were used: monkey kidney cells: protein-free basal or minimum essential Eagle's medium; ERK cells: Earle's solution with 5% calf serum, 5% lactalbumin hydrolysate, and 5% tryptose phosphate broth; HEL cells: reinforced Eagle's medium with 1% fetal calf serum; HeLa cells: Eagle's medium with 5% chicken serum.

Based on data in Eggers and Tamm, 1961a, b; Tamm and Eggers, 1962.

A set of representative results is shown in Fig. 6. The viruses which have been examined may be grouped into four groups, as recorded in Table 2. It is apparent that the cytopathic effects of many HBB-sensitive viruses are completely suppressed at the lowest concentration of inhibitor used, i.e. 98 μM. However, 98 μM HBB inhibits other viruses only for a period of time, after which escape or breakthrough occurs. Such viruses may be completely suppressed by 219 or 493 μM, or they may be only

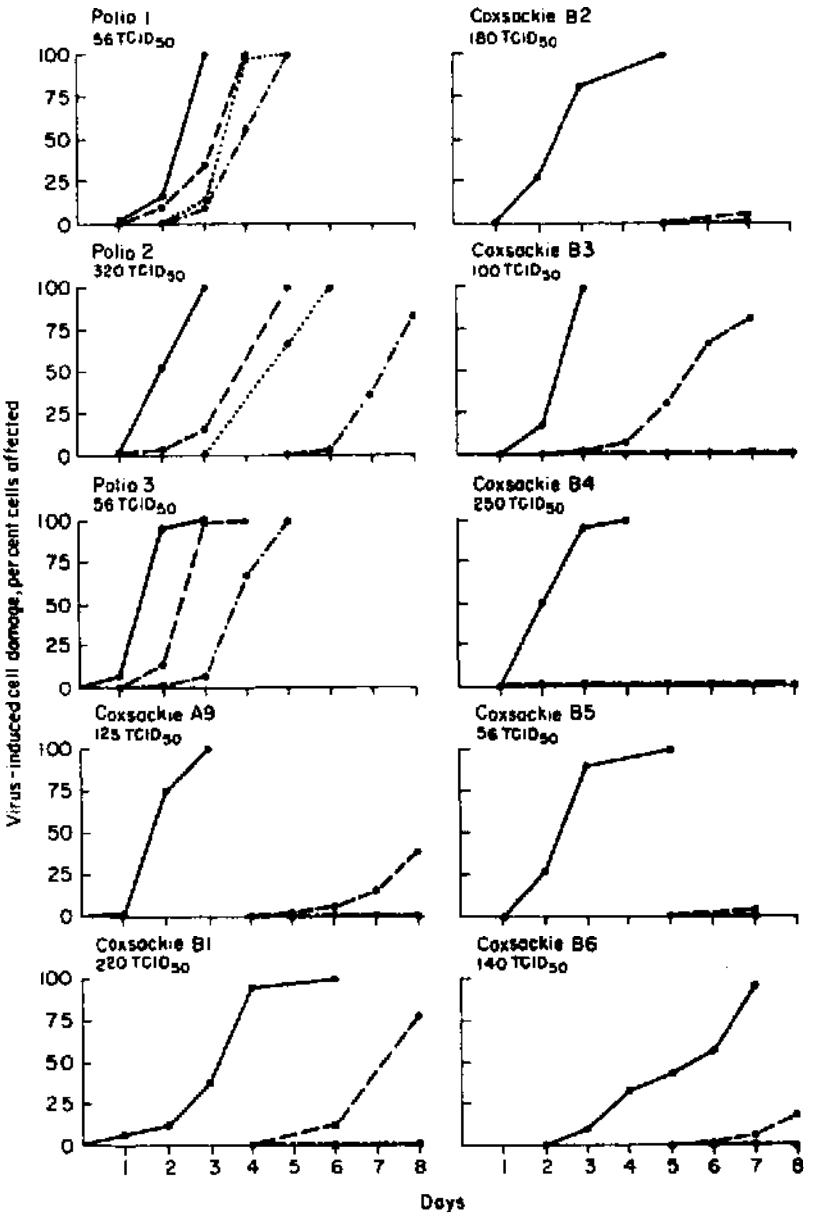


FIG. 6. Inhibition of the cytopathic effects of picornaviruses by HBB in primary cultures of monkey kidney cells. Concentration of HBB: ——— none; - - - - - 98 μM; ····· 219 μM; –·–·– 493 μM. (From Eggers and Tamm, 1961a).

temporarily inhibited even at the highest concentration used. Finally, the development of the cytopathic effects of numerous viruses is not affected by HBB at any of the concentrations used.

On the basis of these findings, it was proposed (Eggers and Tamm, 1961a) that a virus may be considered sensitive to HBB if in the presence of 219 μM HBB the extent of virus-induced cell damage is less than 25% of that in untreated control cultures on any day after infection. It was noted subsequently (Tamm and Eggers, 1962a) that for a qualitative determination of whether a virus is sensitive or insensitive to inhibition by HBB, a single concentration of HBB, such as 50 μg/ml (223 μM), is sufficient. The day when the determination is made would depend on the virus: with rapidly multiplying viruses, 1–2 days of incubation are sufficient; with slower ones, 3–5 days may be required.

The mechanism of escape from inhibition on prolonged incubation requires comment. The available evidence indicates that the virus which replicates in the continued presence of HBB is resistant to inhibitor, or requires HBB for its replication (*vide infra*). Usually the mutant virus populations which emerge are resistant to HBB, but on occasion dependent virus populations have been isolated. Thus, the curves shown in Fig. 6 cannot be interpreted as reflecting in a simple and direct way the sensitivity of the replication process, specifically the synthesis of the RNA of different viruses (*vide infra*), to inhibition by HBB. Rather, such curves may mainly indicate differences in the frequency of mutations from sensitivity to resistance or dependence. The resistance of different mutants to HBB varies over a wide range.

These considerations have an important corollary which bears on the techniques used to determine sensitivity of viruses to HBB. If resistant particles were present in the virus inoculum or if resistant genomes arose soon after inoculation of cells, then the replication and spread of resistant virus in treated cultures might mask the presence of even a substantial number of sensitive virus particles. Such a virus strain might be regarded as HBB-resistant, if examined by a technique which does not permit the determination of the sensitivity of individual particles in the inoculum or of the individual clones of virus arising from the inoculated particles. Only a few attempts have been made to determine whether resistant particles are present in stocks of virus strains such as those used in experiments summarized in Table 2. In a common laboratory stock of the HBB-sensitive coxsackievirus A9, the proportion of resistant particles has been shown to be smaller than 1 in 10,000 (Tamm and Eggers, 1963a). This has been determined by plating the virus in the presence or absence

of HBB and counting the plaques. Similar experiments have not been done with viruses such as coxsackievirus A21 or the polioviruses, which escape from the inhibitory effect of HBB much sooner than coxsackievirus A9.

The results summarized in Table 2 indicate that most of the human enteroviruses are sensitive to inhibition by HBB. This includes most of the echoviruses, all six types of coxsackie B virus, all three types of poliovirus, and some coxsackie A virus types. Among polioviruses, both virulent and attenuated strains are sensitive to HBB. No relationship has been found between attenuation of the strains and sensitivity to HBB; attenuated strains of either type 1 or type 2 virus have been found to be only slightly inhibited, whereas virulent strains vary considerably in their response to HBB (Eggers and Tamm, 1961a).

Echoviruses 22 and 23, which are insusceptible to HBB, cause characteristic cytopathic effects in monkey kidney cells which set them apart from other enteroviruses.

It appears that coxsackie A viruses, as a group, may be insusceptible to HBB. The HBB-sensitive coxsackie virus A9 has been considered to be related to the echo group; and coxsackie A21, which is also sensitive to HBB, is distinguished from other coxsackie A viruses by the fact that it causes respiratory infections of man. However, Hamparian *et al.* (1963) have reported that coxsackievirus A21 is insensitive to HBB in HeLa cells. The basis for the difference in results with coxsackievirus A21 is not clear.

It has been known since the early work (Eggers and Tamm, 1961a) that different strains of an HBB-susceptible virus may vary quantitatively in their sensitivity to inhibition by the compound. However, qualitatively, field strains of picornaviruses resemble the prototype strains in their susceptibility to HBB. Fifty field strains, isolated in widely different geographic locations and at different times, have been compared with respective prototype strains (Eggers and Tamm, 1961b) on the basis of inhibition of virus cytopathic effects by HBB. Thirty-eight presumably susceptible viruses were examined, belonging to poliovirus types 1 and 2; coxsackievirus A type 9; coxsackievirus B types 1–5; and echovirus types 6, 7, 9, 11, 12, 18 and 20. All these strains were susceptible to HBB but there was considerable quantitative variation in the degree of susceptibility. Markedly susceptible polio 1 and echo 20 virus strains were encountered, although the prototype strains have shown only slight sensitivity. Of the twelve presumably insusceptible viruses examined, including echovirus types 22 and 23 and strains of reovirus 3, none was inhibited by HBB. Thus without any exceptions, all 50 field strains examined behaved qualitatively like the prototype strains.

In studies with field strains, HBB has been helpful in detecting mixtures of unrelated viruses (Eggers and Tamm, 1961b). Such mixtures may not be recognized otherwise because, in the absence of HBB, the insusceptible virus may be overgrown by a faster multiplying enterovirus.

As noted in Table 2, representative members of three RNA virus groups, i.e. reo, arbo, and myxo, have been found to be insusceptible to inhibition by HBB. Members of three DNA virus groups, i.e. adeno, herpes and pox, also are insensitive.

It has been reported (Yamazi *et al.*, 1963) that HBB at high concentrations, such as 328 μM, causes some inhibition of the cytopathic effects of Edmonston and Sugiyama strains of measles virus in FL and HeLa cell cultures, but that the reproducibility of the effects is poor. Very small inocula were used in this study. The significance of the findings with measles virus, an agent which has tentatively been assigned to the myxovirus group, is not clear.

HUMAN RHINOVIRUSES

In the earlier studies in several different laboratories most rhinoviruses were found to be insensitive to inhibition by 200–220 μM HBB. These findings are summarized in Table 3 in the column of inhibition results on the left. Of a total of 27 types examined, only two, i.e. types 14 and 26, proved sensitive to HBB; thus, 93% of the types were insensitive. Kisch *et al.* (1964) have reported that the HBB-sensitive type 14 virus strain (1059) is resistant to the action of trypsin, whereas the HBB-insensitive type 13, 15, 16 and 17 viruses (strains 353, 1734, 11757 and 33342) are inactivated by trypsin.

More recently Gwaltney (1968) has examined the effects of 223 μM HBB on the multiplication of 26 rhinoviruses, of which 18 types had been examined previously in other laboratories (Table 3). Types 14 and 26, which were found sensitive by Kisch *et al.* (1964) and Hamre *et al.* (1964), also proved sensitive in Gwaltney's laboratory (1968). Nine of the 18 types were not sensitive in any laboratory; these included types 1A, 1B, 2, 15, 17, 29, 31, 33 and 35. Gwaltney found, however, that 6 other types were slightly to markedly sensitive and that one type gave inconsistent results; other laboratories had found all these 7 types insusceptible. The reasons for the discrepancies are not entirely clear; however, differences in conditions of the experiments and in criteria of inhibition are possible causes. Gwaltney's (1968) comparison of sensitivities of rhinoviruses in WI-38 and HeLa cells indicates that, at the level of insensitivity or marked

TABLE 3. COMPARISON OF RESULTS OF DETERMINATIONS OF SENSITIVITY OF RHINOVIRUSES TO 200–223 μM HBB (Based on Inhibition of Virus Cytopathic Effects)

Type	Strain	Inhibition	Conc. of HBB, μM	Cell type	Reference	Inhibition by 223 μM HBB in WI-38 cells (Gwaltney, 1968)
1A	2060	−*	223	HEL	Tamm and Eggers (1962)	−
1B	B632	−	223	HEL	Tamm and Eggers (1962)	−
2	HGP	−*	219	MKC	Eggers and Tamm (1961b)	−
3	FEB	−*	219	HeLa	Hamparian *et al.* (1963)	+
4	SF748					−
6	SF1349					−
7	11	−	219	HeLa	Hamparian *et al.* (1963)	
11	SF747					±
13	353	−	200	WI-26	Kisch *et al.* (1964)	±
14	1059 or	±	200	WI-26	Kisch *et al.* (1964)	
	101-1	+	200	HEL	Hamre *et al.* (1964)	±
15	1734	−	200	WI-26	Kisch *et al.* (1964)	−
16	11757	−	200	WI-26	Kisch *et al.* (1964)	Inconsistent
17	33342	−	200	WI-26	Kisch *et al.* (1964)	−
18	5,986-CV 17					+
21	CH51					±
22	203-F	−	200	HEL	Hamre *et al.* (1964)	
23	SF1322					−
24	147-H	−	200	HEL	Hamre *et al.* (1964)	
26	127-1	+	200	HEL	Hamre *et al.* (1964)	+
27	SF274					+
28	6,101-CV 29	−	200	HEL	Hamre *et al.* (1964)	
29	179-E	−	200	HEL	Connelly and Hamre (1964)	−
30	106-F	−	200	HEL	Connelly and Hamre (1964)	± or trace

TABLE 3 (*cont.*)

Type	Strain	Inhibition	Conc. of HBB, μM	Cell type	Reference	Inhibition by 223 μM HBB in WI-38 cells (Gwaltney, 1968)
31	140-F	–	200	HEL	Connelly and Hamre (1964)	–
32	363	–	200	WI-26	Webb *et al.* (1964)	trace
33	1200	–	200	WI-26	Webb *et al.* (1964)	–
34	137-3	–	200	HEL	Hamre *et al.* (1964)	+
35	164-A	–	200	HEL	Hamre *et al.* (1964)	–
36	342-H	–	200	HEL	Hamre *et al.* (1964)	
37	151-1	–	200	HEL	Hamre *et al.* (1964)	
38	201-33	–	200	HEL	Hamre *et al.* (1964)	
39	SF299					Inconsistent
40	184-E	–	200	HEL	Hamre *et al.* (1964)	
41	137-F	–	200	HEL	Hamre *et al.* (1964)	±
51	313-G	–	200	HEL	Hamre *et al.* (1964)	

* Hamparian *et al.* (1963) obtained variable results in WI-26 cells with types A1, 2, 3, and also 12, but in HeLa cells consistent results were obtained showing no inhibition of types 2, 3, and 7.

The following media were used: HEL cells: reinforced Eagle's medium with 1% fetal calf serum or equal volumes of Eagle's minimum essential medium and medium 199 with 1% calf serum; monkey kidney cells: protein-free Eagle's minimum essential medium; HeLa cells; Eagle's medium with 5% calf serum or less; WI-26: Eagle's minimum essential medium with 2% calf serum; WI-38: equal volumes of Eagle's minimum essential medium and medium 199 with 2% fetal calf serum.

sensitivity to HBB, the results obtained in different cell types are in good agreement. However, when there is only a low degree of sensitivity in one cell type, the correlation with results in a second cell type may be poor. In the studies of Hamparian *et al.* (1963), WI-26 cells gave variable results, whereas HeLa cells gave more consistent results.

Gwaltney (1968) has also demonstrated considerable quantitative differences in sensitivity to HBB among strains of a single type, i.e. type 7 rhinovirus; all strains, however, are sensitive. These findings are in line with results obtained with enteroviruses (Eggers and Tamm, 1961a). With a generally insensitive rhinovirus type, such as type 24, Gwaltney (1968) has observed that some strains may at times show borderline behaviour, and appear to be slightly inhibited.

Results of extended studies of inhibition of many rhinoviruses by HBB and D-HBB·HCl have demonstrated that these viruses respond to the effects of HBB in a complex pattern (Gwaltney, 1968). In the commonly used preparations of HBB, D- and L-HBB are present in nearly equimolar amounts in the form of free bases. Of the two optical isomers, D-HBB·HCl is 2.5–3 times more active than L-HBB·HCl (Kadin *et al.*, 1964; see section 3). The hydrochloride salts are highly soluble in aqueous media, whereas the free base, HBB, is not. An equimolar mixture of D-HBB·HCl and L-HBB·HCl possesses an inhibitory activity which is indistinguishable from that of DL-HBB. D-HBB·HCl is approximately 1.5 times more active than DL-HBB.

Gwaltney (1968) has reported that successively higher concentrations of HBB or D-HBB·HCl inhibit a progressively greater number of rhinoviruses examined. Table 4 shows that 115 μM D-HBB·HCl (equivalent to 173 μM HBB) inhibits 30% of rhinovirus types, whereas 192 μM D-HBB·HCl (equivalent to 288 μM HBB) inhibits 57%. Indeed, at even higher concentrations of HBB, the great majority of rhinovirus types are inhibited. Two possibilities exist: (1) inhibition at high concentrations of inhibitor is not virus-specific and results secondarily from toxicity of the compound for cells: (2) inhibition even at high concentrations is virus-specific, and not a result of effects of the compound on cells. The fact that some rhinoviruses, i.e. types 1A and 1B, are not inhibited even at toxic concentrations of D-HBB·HCl, suggests that, whatever the nature of the compound-induced toxic changes at high concentrations, such changes are of no consequence for the replication and cytopathogenicity of rhinoviruses. It is well known that picornaviruses are able to replicate in actinomycin-treated cells in which cell RNA and also protein synthesis is markedly inhibited. If the effects of HBB are virus-specific over a wide

range of drug concentrations, then it follows that there is great heterogeneity among rhinoviruses in specific response to HBB.

Gwaltney has found (1968) that using a more sensitive and quantitative gradient plate plaque reduction method (Kucera and Herrmann, 1966), even type 1A shows inhibition at 184 μM D-HBB·HCl (equivalent to 276 μM HBB), and the highly sensitive type 14 is inhibited by 57 μM D-HBB·HCl (equivalent to 85.5 μM HBB). Failure to demonstrate inhibition of many rhinoviruses using the tube culture technique and 200–223 μM HBB may reflect presence of some relatively resistant particles in the seed virus or their common emergence during incubation of infected cells in the presence of HBB.

TABLE 4. RELATIONSHIP BETWEEN CONCENTRATION OF HBB OR D-HBB·HCl AND PROPORTION OF RHINOVIRUS TYPES INHIBITED

	Concentration		Rhinovirus types		
			No. tested	Inhibited (+ or ±)[a]	
	μg/ml	μM		no.	%
D-HBB·HCl[b]	20	77	6[c]	1	17
D-HBB·HCl[b]	30	115	23[c]	7	30
HBB	50	223	26	11	42
D-HBB·HCl	50	192	53	30	57
HBB	100	447	31	18	58
D-HBB·HCl	100	383	42	33	79
D-HBB·HCl	150	574	38	36	95

[a] Viruses were examined in WI-38 cells in equal volumes of Eagle's medium and medium 199 with 2% fetal calf serum; + refers to marked inhibition of virus cytopathic effects, ± to moderate inhibition.

[b] D-HBB·HCl is 1.5 times more active, on a molar concentration basis, than HBB (Kadin *et al.*, 1964).

[c] Most strains tested had previously shown sensitivity to 192 μM D-HBB·HCl.

Modified from Gwaltney, 1968.

ANIMAL PICORNAVIRUSES

HBB inhibits the multiplication and cytopathic effects of Teschen disease virus and 6 porcine enteroviruses (strains 1, 4, 5, 6, 8 and 9) in swine kidney cells (Dardiri *et al.*, 1964). The inhibitory effect of 125 μM

HBB on virus yield is marked and an even greater effect is obtained with 250 or 500 μM HBB. Removal of HBB after 72 hr leads to increased virus cytopathic effects in cultures infected with Teschen disease virus. Thus, the effect of HBB on virus multiplication is reversible. The compound has no direct inactivating effect on the infectivity of Teschen disease virus particles. Pre-treatment of cells with 500 μM HBB for 24 hr, followed by removal of the compound through three washings, does not affect the ability of the cells to support Teschen disease virus multiplication. At virus-inhibitory concentrations HBB does not cause any changes in the microscopic structure of pig kidney or bovine kidney cells. The effects of guanidine on the multiplication of these swine picornaviruses have not been investigated.

The following bovine enteroviruses are inhibited both by HBB and guanidine: GUP; 51/60; 100/60; and 328/60 (Dinter, 1964). HBB at a concentration of 223 μM causes more marked reduction in virus yield than 1040 μM guanidine. Guanidine inhibits the multiplication of bovine enteroviruses in calf kidney cells when the medium is Eagle's minimum essential medium, but not when a medium is used which contains lactalbumin hydrolysate. The blocking of the virus-inhibitory effect of guanidine by amino-acids and choline is discussed in detail in the chapter which follows.

Unexpected results have been obtained with foot-and-mouth disease virus; while the wild type strains O_3, A_4 and C are not inhibited by either HBB or guanidine, the corresponding attenuated strains are inhibited by guanidine (Dinter, 1964). Quantitatively, inhibition by guanidine of the multiplication of attenuated strains of foot-and-mouth disease virus is less marked than inhibition of bovine enteroviruses. That HBB has no inhibitory effect on the multiplication of foot-and-mouth disease virus has also been demonstrated with type A strain 119 virus, which had been passaged about 90 times in calf-kidney cultures (Dardiri *et al.*, 1964).

The clear-cut inhibition findings with HBB and bovine and porcine enteroviruses are entirely in agreement with those obtained with human enteroviruses. As foot-and-mouth disease virus possesses a number of physical–chemical characteristics which distinguish it from enteroviruses, it is not surprising that the response of foot-and-mouth disease virus to the selective inhibitors is also distinct.

LYMPHOCYTIC CHORIOMENINGITIS VIRUS

HBB inhibits the multiplication of three strains of lymphocytic choriomeningitis (LCM) virus in HeLa cells (Pfau and Camyre, 1968). These

observations are an exception to the rule that HBB does not inhibit the multiplication of viruses in major groups outside of picornaviruses. Lymphocytic choriomeningitis virus is an unclassified virus whose size and ether sensitivity indicate that it is not a picornavirus. The Traub, WCP and CA1371 strains grow without causing cytopathic effects in one line of HeLa cells, but fail to grow in a number of others. These strains of LCM virus can be assayed in mice. Concentrations of HBB as low as 55 μM significantly reduce the amounts of LCM viruses produced in HeLa cells. The control yields reach a peak in 1–2 days and then decline. In HBB-treated cultures, some virus is produced in the presence of 55 or 110 μM HBB, but by the 2nd or 3rd day little infective virus remains, as the small amounts of virus produced probably become thermally inactivated. 219 μM HBB completely suppresses virus multiplication.

HBB at a concentration of 219 μM has no effect on the multiplication of uninfected HeLa cells (Eggers and Tamm, 1961a; Pfau and Camyre, 1968), but it does noticeably slow the division rate of LCM-infected cells between 24 and 48 hr after infection (Pfau and Camyre, 1968). This effect is apparently due to virus inhibition, as the multiplication rate of untreated LCM virus-infected cells is slightly higher than that of uninfected control cells. Thus, by inhibiting LCM virus multiplication, HBB prevents the stimulation of cell division by LCM. HBB has no demonstrable effects on the virus *in vitro* or on its adsorption to cells.

Sensitivity of LCM virus to inhibition by HBB indicates that the action of this compound is not entirely restricted to picornaviruses. It is not known whether HBB inhibits the multiplication of LCM virus by the same mechanism as is involved in the inhibition of picornaviruses, i.e. through an effect on virus RNA synthesis. It is noteworthy that LCM is not inhibited by 700 μM guanidine. Among enteroviruses, no agent has been found whose multiplication is sensitive to HBB but not to guanidine.

STRUCTURE–ACTIVITY RELATIONSHIPS

Of the two optical isomers of HBB, D-HBB·HCl is 2.5–3 times more active and selective than L-HBB·HCl in inhibiting picornavirus multiplication (Kadin *et al.*, 1964). This has been determined quantitatively with coxsackievirus A9 in single-cycle growth experiments in monkey kidney cells. D-HBB·HCl is also more active than L-HBB·HCl against echovirus 6, echovirus 12, and poliovirus 2, as determined in multiple-cycle experiments by observing development of cytopathic effects. D-HBB·HCl, L-HBB·HCl, and DL-HBB show closely similar degrees of toxicity (Kadin

et al., 1964). Concentrations of D-HBB·HCl in excess of 350 μM cause morphological changes in monkey kidney cells (Kadin *et al.*, 1964) or diploid human embryonic lung cells (Gwaltney, 1968); this corresponds to the toxicity of HBB (Tamm *et al.*, 1961; Tamm and Eggers, 1962; Tamm *et al.*, 1969).

The hydroxybenzyl grouping at position 2 in the benzimidazole nucleus is of fundamental importance for the selective virus-inhibiting activity of HBB (Tamm *et al.*, 1961; Tamm and Eggers, 1963a, b). Figure 7 shows that the 2-hydroxymethyl and 2-(α-hydroxyethyl) derivatives of benzimidazole are inactive; furthermore, the 2-benzyl and 2-benzoyl derivatives show much reduced activity and selectivity. The benzo moiety in the benzimidazolyl nucleus in HBB is also critically important, as replacement of the entire benzimidazolyl nucleus with the monocyclic imidazole group results in complete loss of activity (Tamm and Eggers, 1963a; Tamm *et al.*, 1969). The toxicity of 2-(α-hydroxybenzyl)imidazole is similar to that of HBB. Thus, the benzo moiety in HBB specifically imparts virus-inhibitory activity to the molecule.

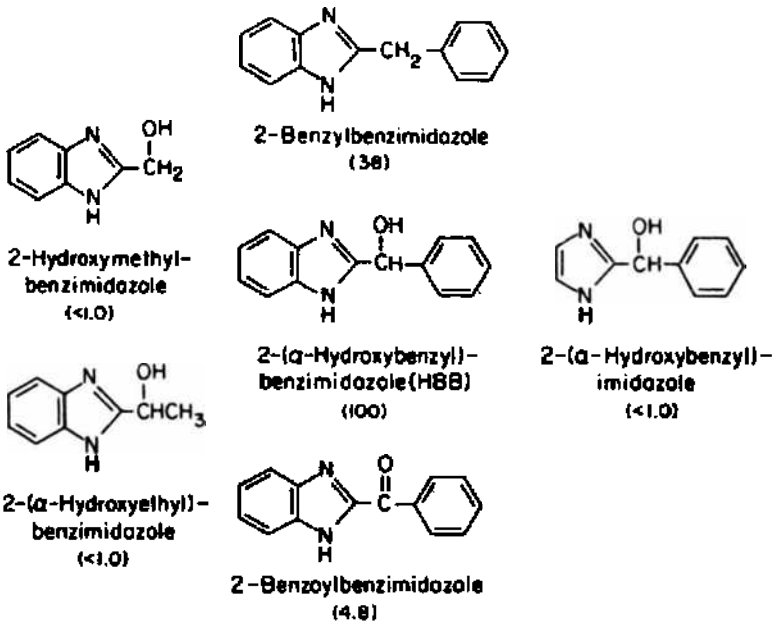


FIG. 7. The structural basis of the picornavirus-inhibitory activity of 2-(α-hydroxybenzyl)benzimidazole (HBB). The activities are expressed relative to the poliovirus-inhibitory activity of HBB, which has been assigned the value of 100. HBB, the reference compound, causes 75% inhibition poliovirus yield at a concentration of 36 μM. Lack of activity of 2-(α-hydroxybenzyl)imidazole was demonstrated with echovirus 6. (Modified and expanded from Tamm and Eggers, 1963b.)

O'Sullivan and Sadler (1961) reported that 2-(*o*-hydroxybenzyl)-benzimidazole inhibits poliovirus 2 (strain P712-ch-2ab) in ERK cells to a similar degree as does HBB. In more quantitative experiments in monkey kidney cells, 98 μM *o*-hydroxy compound has been found to be significantly less active than 98 μM HBB against poliovirus 2 (P712-ch-2ab and MEF1), coxsackievirus A9 (Woods), and echovirus 6 (D'Amori) (Eggers and Tamm, 1961, unpublished observations). The modest virus-inhibitory effect of 2-(*o*-hydroxybenzyl)benzimidazole is however a selective one, as shown by results of experiments with an HBB-resistant variant of coxsackievirus A9, and with echovirus 22, which is insensitive to HBB. Neither HBB (219 μM) nor 2-(*o*-hydroxybenzyl)benzimidazole (493 μM) has any effect on the development of cytopathic changes in cells infected with either of these two viruses.

TABLE 5. POLIOVIRUS-INHIBITORY ACTIVITY AND TOXICITY OF *N*-1-SUBSTITUTED 2-(α-HYDROXYBENZYL) BENZIMIDAZOLES IN ERK CELLS*

Substituent at position *N*-1	Maximum tolerated concentration, μM	Concentration giving 75% inhibition of poliovirus multiplication, μM		
		Type 1 (L Sc, 2 ab)	Type 2 (P712,Ch, 2 ab)	Type 3 (Leon 12 ab)
H	210	160	35	160
β-Methylpropyl	100	13	10	25
Propyl	80	9	7.5	22.5
Phenyl	140	6	5	15
Butyl	60	10	5.5	25
Benzyl	100	20	12.5	25

* The medium was Earle's solution with lactalbumin hydrolysate and papain digest broth.

From O'Sullivan *et al.*, 1967.

Certain 1-substituted derivatives, such as 1-propyl and 1-phenyl-HBB, are highly active and selective in inhibiting poliovirus multiplication (O'Sullivan and Wallis, 1963a; O'Sullivan *et al.*, 1964a, b, 1967). The selective poliovirus-inhibitor activity of 1-alkyl-2-(α-hydroxybenzyl)benzimidazoles increases from the 1-methyl to the 1-propyl compound (O'Sullivan and Wallis, 1963a). The 1-phenyl derivative of HBB is even more active than

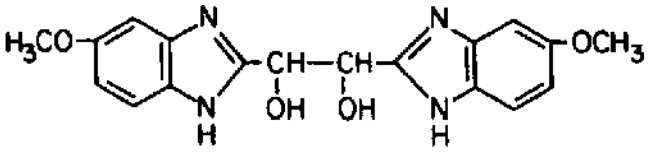
1-propyl-HBB (O'Sullivan *et al.*, 1967). Table 5 summarizes the virus-inhibitory activities of a number of 1-substituted compounds. 1-Phenyl-HBB is 7 times more active than HBB in inhibiting poliovirus 2. Its activity against poliovirus 1 is 27 times greater, and against poliovirus 3, 11 times greater than that of HBB. The quantitative estimation of the selectivity is rendered difficult by the fact that compounds such as 1-phenyl-HBB are poorly soluble in water. The concentrations indicated refer to micromoles of compound dispersed per liter of medium. The toxicities, if based on the amounts of compound actually dissolved, might be significantly greater than the data would indicate. Low solubility might also affect determinations of virus-inhibitory activity, but obviously to a lesser extent, as the selective inhibitors are active against sensitive virus even at very low concentrations.

As for the virus-inhibitory activity of HBB itself, the finding that 35 μM HBB causes 75% inhibition of poliovirus 2 multiplication in ERK cells (see Table 5) compares closely with the earlier demonstration that 36 μM HBB causes 75% inhibition of poliovirus 2 in monkey kidney cells (Tamm *et al.*, 1961).

It has been reported that the D-isomers of 1-methyl, 1-ethyl, 1-propyl and 1-butyl-HBB have no greater protective activities than the DL compounds against poliovirus-induced cytopathic effects (O'Sullivan *et al.*, 1964a). The suggestion has been made that the L-isomers would be expected to behave similarly to the DL-isomers, and that, therefore, the protective action of the 1-alkyl compounds may not be related to the configuration at the α-carbon. This question cannot be settled until the L-isomers are examined.

Five 1,3-dialkylbenzimidazolium iodides have been found to have reduced protective activity against polioviruses (O'Sullivan and Wallis, 1963b). Thus, the effect of quaternization is to reduce the protective action of 1-alkyl derivatives.

O'Sullivan and Wallis (1963a) found that 1,2-*bis*(2-benzimidazolyl)-1,2-ethanediol has slight protective activity against poliovirus 1 in rabbit embryo kidney cells, but none against polioviruses 2 and 3. Several such derivatives have been synthesized and examined by Akihama *et al.* (1968). Again, only slight inhibition of poliovirus 1 multiplication in HeLa cells has been obtained with the parent compound, but 1,2-*bis*-(5[or 6]-methoxy-2-benzimidazolyl)-1,2-ethanediol (Fig. 8) has a marked effect on plaque formation by poliovirus 1; concentrations from 1 to 100 μM all cause complete inhibition. The poliovirus-inhibitory activity has also been determined by performing $TCID_{50}$ titrations in the presence or



1,2-*bis*-[5(or 6)-methoxy-2-benzimidazolyl]-1,2-ethanediol

FIG. 8. The structure 1,2-*bis*-(5[or 6]-methoxy-2-benzimidazolyl)-1,2-ethanediol—the most active inhibitor of poliovirus multiplication reported. (From Akihama *et al.*, 1968.)

absence of varying concentrations of the inhibitor. The results in Table 6 indicate that the minimum effective concentration is between 0.1 and 1.0 μM. This would suggest that 1,2-*bis*-(5[or 6]-methoxy-2-benzimidazolyl)-1,2-ethanediol may be about 10 times more active than 1-phenyl-2-(α-hydroxybenzyl)benzimidazole and of the order of 100 times more active than HBB. It has been reported that the *bis*-benzimidazolyl derivative shows no cytotoxicity at a concentration of 100 μM over the course of 7 days, but that at 1000 μM concentration of inhibitor the cells degenerate.

The poliovirus-inhibitory activity of a series of guanidinobenzimidazole derivatives has also been investigated (Sato *et al.*, 1966). Among these

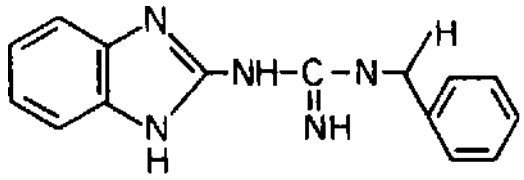
TABLE 6. POLIOVIRUS INHIBITORY ACTIVITY OF 1, 2-*bis*-(5[OR 6]-METHOXY-2-BENZIMIDAZOLYL)-1,2-ETHANEDIOL IN HELA S3 CELLS[a]

Concentration of inhibitor, μM	Poliovirus 1 (Mahoney)		
	$-\log TCID_{50}$		Difference $-\log TCID_{50}$
	Untreated	Treated	
100	9.00	0.75	8.25
10	9.00	1.25	7.75
1	9.00	1.50	7.50
0.1	9.00	8.75	0.25
0.01	9.00	9.00	0.00

[a] The medium was Earle's solution with yeast extract, lactalbumin hydrolysate and calf serum.

From Akihama *et al.*, 1968.

compounds, 2-N^3-phenylguanidinobenzimidazole (Fig. 9) is most effective in reducing both the size and number of plaques produced by poliovirus. Although it is several times more active than HBB, it is also considerably more toxic, and thus less selective than HBB.



2-N^3-phenylguanidinobenzimidazole

FIG. 9. The structure 2-N^3-phenylguanidinobenzimidazole—an active but not highly selective inhibitor of poliovirus multiplication. (From Sato *et al.*, 1966.)

Studies of structure–activity relationships with analogs and derivatives of HBB have been extended to numerous other compounds (Tamm *et al.*, 1969), including a series of new derivatives (Wagner *et al.*, 1962). Echovirus type 6 has been employed in a large study because it is one of the most sensitive viruses to inhibition by HBB (Eggers and Tamm, 1961a). The results of studies with echovirus 6 are summarized in Tables 7 and 8.

The activities and toxicities of derivatives are compared on a numerical basis (Tamm *et al.*, 1969). The experimental technique used can be summarized as follows. Primary monolayer cultures of rhesus monkey kidney cells in screw-cap tubes, containing approximately 2.5×10^5 cells per tube, are inoculated with 500 $TCID_{50}$ of echovirus type 6 (D'Amori strain) per culture. The medium used is Eagle's minimum essential medium (Eagle, 1959). Each compound is employed at a series of concentrations. Appropriate uninfected or untreated controls are included in each experiment. The cultures are held stationary at 36°C. Microscopic observations of both cytotoxic and virus cytopathic effects are made on the 3rd and 6th days of incubation. Cell damage is expressed in terms of the proportion of cells showing marked changes, using a scale from 0 to 4 plus, with 1+ corresponding to 25%, 2+ to 50%, 3+ to 75%, and 4+ to 100% involvement of monolayer.

Extent of cytotoxic changes is plotted against the concentration of compound and a line fitted to the experimental points. From this line the concentration of compound which causes minimal cytotoxic changes is determined. A minimal cytotoxicity endpoint permits comparison of the toxicity of many inhibitors, including those which are poorly soluble.

TABLE 7. INHIBITION OF CYTOPATHIC EFFECTS OF ECHOVIRUS 6 (D'AMORI) BY COMPOUNDS RELATED TO 2-(α-HYDROXYBENZYL)BENZIMIDAZOLE, AND TOXICITY IN MONKEY KIDNEY CELLS

Group	No.	Compound Designation	Mol. wt.	75% protective concentration, μM		Minimal toxic concentration, μM		Selectivity ratio, Minimal toxic conc. / 75% protective conc.	
				3rd day	6th day	3rd day	6th day	3rd day	6th day
	1	2-(α-Hydroxybenzyl)benzimidazole (HBB)	224	37	90	780	460	21	5.1
A	2	2-(α-Methyl-α-hydroxybenzyl)benzimidazole	238	14	35	460	300	33	8.6
	3	2-(α-Ethyl-α-hydroxybenzyl)benzimidazole	252	20	37	270	130	14	3.5
	4	2-(α-Isopropyl-α-hydroxybenzyl)benzimidazole	266	130	220	>490	350	>3.8	1.6
	5	2-(α-Methallyl-α-hydroxybenzyl)benzimidazole	278	220	>490	>490	450	>2.2	—
	6	2-(α-Carbethoxymethyl-α-hydroxybenzyl)-benzimidazole	310	>490	>490	>490	490	—	—
	7	Ammonium salt of 2-(α-carboxymethyl-α-hydroxybenzyl)benzimidazole	299	>490	>490	>490	>490	—	—
	8	2-(α-Phenyl-α-hydroxybenzyl)benzimidazole	300	>490	>490	>490	>490	—	—
B	9	5-Trifluoromethyl-2-(α-methyl-α-hydroxybenzyl)-benzimidazole	306	7	9	31	14	4.4	1.6
	10	5-Trifluoromethyl-2-(α-hydroxybenzyl)-benzimidazole	292	7	15	90	26	13	1.7
	11	5-Phenyl-2-(α-hydroxybenzyl)benzimidazole	300	19	—	50	13	2.6	—
	12	5-Methyl-2-(α-hydroxybenzyl)benzimidazole	238	40	170	310	150	7.8	0.88
	13	5-Nitro-2-(α-hydroxybenzyl)benzimidazole	269	80	—	110	26	1.4	—
	14	5-Methoxy-2-(α-hydroxybenzyl)benzimidazole	254	220	—	270	160	1.2	—
	15	4-Ethyl-2-(α-hydroxybenzyl)benzimidazole	252	>490	—	>490	140	—	—

TABLE 7 (*cont.*)

Group	No.	Compound Designation	Mol. wt.	75% protective concentration, μM		Minimal toxic concentration, μM		Selectivity ratio, Minimal toxic conc. / 75% protective conc.	
				3rd day	6th day	3rd day	6th day	3rd day	6th day
C	16	1-Methyl-2-(α-hydroxybenzyl)-5-trifluoromethylbenzimidazole	306	14	18	210	48	15	2.7
	17	1-Methyl-2-(α-methyl-α-hydroxybenzyl)-benzimidazole	252	20	37	>490	140	>25	3.8
	18	5-Trifluoromethyl-1-methyl-2-(α-methyl-α-hydroxybenzyl)benzimidazole	320	22	37	380	110	17	3.0
	19	1-Methyl-2-(α-hydroxybenzyl)benzimidazole	238	42	160	470	130	11	0.81
	20	Sodium salt of 1-carboxymethyl-2-(α-methyl-α-hydroxybenzyl)benzimidazole	318	>490	>490	>490	>490	—	—
D	21	2-(2-Thienyl-hydroxymethyl)benzimidazole	230	42	100	570	260	14	2.6
	22	2-(*p*-Chloro-α-hydroxybenzyl)benzimidazole	259	44	—	65	12	1.5	—
	23	2-(β-Benzylthio-α-hydroxyethyl)benzimidazole	284	60	—	200	66	3.3	—
	24	2-(2-Furylhydroxymethyl)benzimidazole	214	—	—	98	58	—	—
	25	2-(α-Hydroxy-α-cyclohexylmethyl)benzimidazole	230	>490	>490	>490	390	—	—
	26	2-(α-Hydroxy-*m*-nitrobenzyl)benzimidazole	269	>490	>490	>490	>490	—	—
E	27	2-(α-Methoxybenzyl)benzimidazole	238	34	38	340	180	10	4.7
	28	2-(α-Acetoxybenzyl)benzimidazole	266	39	120	>490	460	13	3.8
	29	1-Acetyl-2-(α-acetoxybenzyl)benzimidazole	308	122	—	240	150	2.0	—

From Tamm *et al.*, 1969.

TABLE 8. ECHOVIRUS 6 INHIBITORY ACTIVITY AND SELECTIVITY IN MONKEY KIDNEY CELLS OF COMPOUNDS RELATED TO 2-(α-Hydroxybenzyl)benzimidazole

VIA = virus inhibitory activity S = selectivity	No.	Compound Designation	Relative activity		Relative toxicity		Relative selectivity	
			3rd day	6th day	3rd day	6th day	3rd day	6th day
Reference VIA and S	1	2-(α-Hydroxybenzyl)benzimidazole (HBB)	1.0	1.0	1.0	1.0	1.0	1.0
VIA increased— S retained	2	2-(α-Methyl-α-hydroxybenzyl)benzimidazole	2.6	2.6	1.7	1.5	1.6	1.7
VIA increased— S reduced (compounds listed in order of decreasing VIA)[a]	10	5-Trifluoromethyl-2-(α-hydroxybenzyl)-benzimidazole	5.3	6.0	8.7	18	0.62	0.33
	9	5-Trifluoromethyl-2-(α-methyl-α-hydroxy-benzyl)benzimidazole	5.3	10	25	33	0.21	0.31
	16	1-Methyl-2-(α-hydroxybenzyl)-5-tri-fluoromethylbenzimidazole	2.6	5.0	3.7	9.6	0.71	0.53
	11	5-Phenyl-2-(α-hydroxybenzyl)-benzimidazole	1.9	—	16	35	0.12	—
	17	1-Methyl-2-(α-methyl-α-hydroxybenzyl)-benzimidazole	1.8	2.4	—	3.3	—	0.74
	3	2-(α-Ethyl-α-hydroxybenzyl)benzimidazole	1.8	2.4	2.9	3.5	0.67	0.69
	18	5-Trifluoromethyl-1-methyl-2-(α-methyl-α-hydroxybenzyl)benzimidazole	1.7	2.4	2.0	4.2	0.81	0.59
VIA retained— S retained or reduced (compounds listed in order of increasing toxicity)[b]	28	2-(α-Acetoxybenzyl)benzimidazole	0.95	0.75	—	1.0	—	0.74
	21	2-(2-Thienyl-hydroxymethyl)benzimidazole	0.88	0.90	1.4	1.8	0.67	0.51
	27	2-(α-Methoxybenzyl)benzimidazole	1.1	2.4	2.3	2.6	0.48	0.92
	12	5-Methyl-2-(α-hydroxybenzyl)benzimidazole	0.92	0.53	2.5	3.1	0.37	0.17
	19	1-Methyl-2-(α-hydroxybenzyl)benzimidazole	0.88	0.56	1.7	3.5	0.52	0.16
	22	2-(*p*-Chloro-α-hydroxybenzyl)benzimidazole	0.84	—	12	38	0.07	—

TABLE 8 (*cont.*)

VIA = virus inhibitory activity S = selectivity	No.	Compound Designation	Relative activity		Relative toxicity		Relative selectivity	
			3rd day	6th day	3rd day	6th day	3rd day	6th day
VIA reduced—S reduced (compounds listed in order of decreasing VIA)[a]	23	2-(β-Benzylthio-α-hydroxyethyl)-benzimidazole	0.62	—	3.9	7.0	0.16	—
	13	5-Nitro-2-(α-hydroxybenzyl)benzimidazole	0.46	—	7.1	18	0.07	—
	29	1-Acetyl-2-(α-acetoxybenzyl)benzimidazole	0.30	—	3.2	3.1	0.09	—
	4	2-(α-Isopropyl-α-hydroxybenzyl)-benzimidazole	0.28	0.41	—	1.3	—	0.31
	14	5-Methoxy-2-(α-hydroxybenzyl)-benzimidazole	0.17	—	2.9	2.9	0.06	—
	5	2-(α-Methallyl-α-hydroxybenzyl)-benzimidazole	0.17	—	—	1.0	—	—
No selective VIA (compounds listed in order of increasing toxicity)[b]	6	2-(α-Carbethoxymethyl-α-hydroxybenzyl)-benzimidazole	—	—	—	0.94	—	—
	25	2-(α-Hydroxy-α-cyclohexylmethyl)-benzimidazole	—	—	—	1.2	—	—
	15	4-Ethyl-2-(α-hydroxybenzyl)benzimidazole	—	—	—	3.3	—	—
	24	2-(2-Furylhydroxymethyl)benzimidazole	—	—	8.0	7.9	—	—
No VIA—non-toxic compounds	7	Ammonium salt of 2-(α-carboxymethyl-α-hydroxybenzyl)benzimidazole	—	—	—	—	—	—
	8	2-(α-Phenyl-α-hydroxybenzyl)benzimidazole	—	—	—	—	—	—
	20	Sodium salt of 1-carboxymethyl-2-(α-methyl-α-hydroxybenzyl)benzimidazole	—	—	—	—	—	—
	26	2-(α-Hydroxy-*m*-nitrobenzyl)benzimidazole	—	—	—	—	—	—

[a] Order of listing based on 3rd day determinations.
[b] Order of listing based on 6th day determinations.
From Tamm *et al.*, 1969.

The extent of virus cytopathic effects in the presence of compound is expressed as a percentage value of cytopathic effects in untreated infected controls and plotted against the concentration of the compound. The concentration which causes 75% inhibition of virus cytopathic effects is determined from the curve. Selectivity is estimated in terms of the ratio

$$\frac{\text{minimal toxic concentration}}{\text{75\% inhibitory concentration}}$$

In Table 7 compounds are grouped on the basis of chemical structure and listed within each group in accordance with increasing virus-inhibitory activity. In this table the results are expressed in terms of the micromolar concentrations of compounds required for 75% protection of cultures against virus cytopathic effects or to cause minimal toxic effects, and selectivity ratios are given for each compound. In Table 8 the compounds are grouped on the basis of virus-inhibitory activity and selectivity, and the results expressed relative to the activity, toxicity, and selectivity of HBB. For cross-reference, the compounds have been numbered from 1 to 29.

The most significant changes in virus-inhibitory activity in this study involve replacement of the hydrogen atom on the α-position of the 2-(α-hydroxybenzyl) moiety of HBB (compounds 2–8). The α-methyl derivative (compound 2) is more than twice as active as HBB while its toxicity is less than twice that of the reference compound. 2-(α-Methyl-α-hydroxybenzyl)benzimidazole (2) is the only derivative in this series which is not only more active, but also somewhat more selective than HBB. The α-ethyl derivative (3) also is more active, but at the same time less selective than HBB. It has been reported (O'Sullivan and Wallis, 1963a) that with polioviruses the α-methyl and related derivatives give less protection to infected cells than HBB itself or derivatives not possessing the fourth substituent at the α-carbon. This may reflect differences between polio and other enteroviruses in sensitivity to inhibitors. Such differences have been noted in sensitivity to inhibition by HBB and guanidine (Tamm and Eggers, 1962).

Decreased activity and selectivity are observed when three- and four-carbon unit aliphatic substituents, such as isopropyl (4) and methallyl (5), are substituted for the α-hydrogen atom of the hydroxybenzyl moiety. The α-phenyl derivative (8) and two derivatives with oxygen-containing substituents, i.e., α-carbethoxymethyl (6) and α-carboxymethyl (7), are all substantially less active.

The enhanced virus-inhibitory activity obtained by substituting methyl or ethyl groups (2 and 3) for hydrogen at the α-carbon atom of the hydroxybenzyl moiety suggests that decreased acidity of the carbinol function may be of importance for activity. This concept is also supported by the fact that the 2-(α-methoxybenzyl) derivative (27) is active. The substantial losses in activity observed when large substituents (8) are introduced at the α-carbon atom suggest unfavourable steric effects of such substituents.

The derivatives in this series showing highest virus-inhibiting activity are 5-trifluoromethyl-2-(α-hydroxybenzyl)benzimidazole (10) and the corresponding α-methyl-substituted compound (9), both containing an electron-withdrawing substituent at position 5 in the benzenoid ring. However both inhibitors also show considerably increased toxicity and are thus less selective than HBB. The effect of other electron-withdrawing substituents at the 5-position of the benzimidazolyl moiety is variable. The phenyl substituent (11) produces a moderate increase in activity but this is associated with a marked increase in toxicity, while the nitro substituent (13) causes a reduction in activity which is also associated with increased toxicity.

The introduction of electron-donating groups at the 4- or 5-position of the benzimidazolyl moiety produces deleterious effects. For example, 5-methyl-2-(α-hydroxybenzyl)benzimidazole (12) is as active as HBB but much less selective. It shows little protective effect against polioviruses at maximal tolerated concentration (O'Sullivan and Wallis, 1963a).

The introduction of a methyl group at *N*-1 of the benzimidazolyl moiety, while lacking in favorable effects, does not destroy echovirus-inhibitory activity (16–19), a result also obtained with polioviruses (O'Sullivan and Wallis, 1963a). Furthermore, as discussed above, the 1-ethyl, 1-propyl, 1-butyl, and 1-phenyl derivatives show considerably increased poliovirus-inhibitory activity and selectivity (O'Sullivan and Wallis, 1963a; O'Sullivan *et al.*, 1967). These results suggest that the activity of HBB and its analogs is not dependent upon their biosynthetic conversion to a nucleoside by condensation at *N*-1 with ribose or deoxyribose.

Replacement of the phenyl group of the hydroxybenzyl moiety of HBB with other cyclic groups produces no desirable effects on activity or toxicity (21–26). Replacement by a 2-thienyl (21), *p*-chlorophenyl (22), or β-benzylthio (23) group has relatively little effect on activity and the 2-thienyl group (21) does not produce a marked effect on toxicity, but the toxicity of the other two compounds (22 and 23) is much increased. The

hydrochloride of 2-(*p*-chloro-α-hydroxybenzyl)benzimidazole lacks protective activity against polioviruses (O'Sullivan and Wallis, 1963a). When the phenyl group of the hydroxybenzyl moiety is replaced by a deactivated aromatic ring such as *m*-nitrophenyl (26) or a nonaromatic ring such as cyclohexyl (25), activity is destroyed. These results emphasize the importance of the aromaticity of the 2-substituent relative to the steric or geometric effect.

Intramolecular hydrogen bonding involving the hydroxyl group cannot occur in the α-methoxybenzyl (27) or α-acetoxybenzyl (28) derivative of benzimidazole, and yet both are as active as HBB. Clearly, formation of such intramolecular hydrogen bonds is not essential for virus-inhibitory activity (Tamm and Eggers, 1963a). 2-(α-Methoxybenzyl)benzimidazole also is active against polioviruses at a level similar to that of HBB (O'Sullivan *et al.*, 1965).

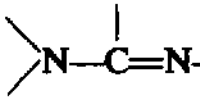
There is much support for the view that the aralkyl nature of the 2-substituent is essential for activity in the benzimidazolyl series since no virus-inhibitory activity is observed with 2-(2-thienyl)-, 2-(2-furyl)-, or 2-phenylbenzimidazole (Tamm *et al.*, 1969).

Not surprisingly, the introduction of a benzothiazole nucleus in place of the benzimidazolyl nucleus in HBB does not markedly affect the virus-inhibitory activity. As predictable from knowledge of the activities of HBB (1) and its α-methyl derivative (2), the α-methyl derivative of 2-(α-hydroxybenzyl)benzothiazole is approximately twice as active as the parent compound (Tamm *et al.*, 1969). Both benzothiazole derivatives are less selective than the benzimidazole derivatives. In contrast to the experience in the benzimidazole series, the complete replacement of the hydroxybenzyl moiety by phenyl in the benzothiazole series results in a considerable loss of virus-inhibitory activity, but not in complete inactivation.

As a perspective, it is evident from the extensive studies with echovirus 6 that HBB itself is a remarkably effective compound with respect to both its degree of virus-inhibitory activity and its favorable selectivity ratio (Tamm *et al.*, 1969). In these studies, only one compound, 2-(α-methyl-α-hydroxybenzyl)benzimidazole (2), has shown both increased virus-inhibitory activity and selectivity. Although some other analogs possess increased virus-inhibitory activity, the increase is more than offset by increased toxicity. 5-Trifluoromethyl-2-(α-hydroxybenzyl)benzimidazole (10) is the most active compound in the group of derivatives investigated with echovirus 6, but it is significantly less selective than HBB.

It would appear that the mechanism of the inhibitory activity of HBB is dependent upon the structural characteristics of the molecule *per se*.

The weight of evidence favors the concept that one (or both) of the nitrogen atoms, rather than the hydroxyl group of HBB, is essential for the binding of HBB to its target. The over-all geometry of the entire HBB molecule from the 5- and 6-positions of the benzimidazole moiety to the *p*-position of the phenyl moiety appears to be of importance for activity. The electron-withdrawing capacity of the entire molecule is reasonably essential. The spatial relationship of the planar phenyl moiety to the planar benzimidazole moiety is apparently important.

HBB and guanidine are quite different with respect to molecular size, aromaticity, steric points, number of nitrogen atoms and basicity. Yet because of similarities in their actions it seems likely that these compounds have a feature in common which is involved in the specific virus-inhibiting activity (Tamm and Eggers, 1963a). The  sequence in both may be such a feature. Though this may be an essential feature in HBB, it is clear that this sequence is not of itself sufficient for biological activity in HBB since 2-(α-hydroxybenzyl)imidazole and 2-hydroxymethylbenzimidazole are both completely inactive. It thus appears that the overall configuration of HBB is of critical importance.

EFFECTS ON NORMAL CELLS

Most of the studies of the effects of HBB on normal cells or on virus multiplication have been carried out in primary cultures of monkey kidney cells. As already stated above, 780 μM HBB causes minimal morphological changes in monkey kidney cells after 3 days of incubation in protein-free Eagle's medium, and similar effects are observed after 6 days with 460 μM HBB (Tamm *et al.*, 1969). The degree of morphological changes in cells varies with the batch of cultures (Eggers and Tamm, 1961a). The changes are minimal in cultures of excellent quality, but in cultures in which untreated cells show some granular changes, the cytotoxicity due to 493 μM HBB may become moderate to marked on prolonged incubation in protein-free Eagle's medium. At the commonly used concentration, i.e., 219 μM or 49 μg/ml, no such changes can usually be detected. Occasionally, however, slight changes are observed after 5 to 7 days of incubation. Similar results have been obtained with ERK cell cultures. If the medium is not changed frequently, HeLa cells in the presence of 219 μM HBB degenerate earlier and more extensively than do untreated cells.

In Table 9 are summarized the conditions under which HBB has shown no effects on metabolic activities of monkey kidney cells in protein-free Eagle's medium (Eggers and Tamm, 1961a, 1962a). In addition, 219 μM HBB has no effect on the rate of cell RNA or protein synthesis as measured

TABLE 9. CONDITIONS UNDER WHICH HBB HAS SHOWN NO EFFECTS ON METABOLIC ACTIVITIES OF MONKEY KIDNEY CELLS

Cell culture	Metabolic process	Duration (hr)	Concentration of HBB, μM
Suspension	Cumulative O_2 uptake	3	98, 219, 493
Suspension	Glucose utilization	3	98, 219, 493
Suspension	Lactic acid production	3	98, 219, 493
Monolayer	Glucose utilization	3	98, 219, 493
Monolayer	Lactic acid production	24	98, 219, 493
Suspension	Adenosine-8-^{14}C uptake into RNA	3	19.5, 98, 493
Suspension	L-Alanine-^{14}C uptake into proteins	3	19.5, 493
Monolayer	Uridine-^{3}H uptake into RNA	6.5	98, 219

† Cells were treated with HBB for 6.5 hr; uridine-^{3}H was present during the last 1.5 hr.
From Eggers and Tamm, 1961a; data on the last line are from Eggers and Tamm, 1962a.

in pulse-labeling experiments with ^{3}H-uridine and ^{3}H-DL-leucine (Bablanian and Tamm, 1963, unpublished results). However, in similar experiments with a strain of human embryonic lung (HEL) cells, the rates of incorporation of the tritiated precursors are reduced by approximately 25% after 4 hr of treatment with HBB. In experiments with HEL cells, reinforced Eagle's medium was used (Bablanian *et al.*, 1965a).

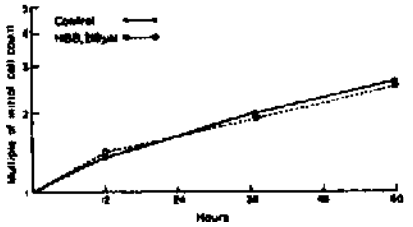


FIG. 10. Multiplication of rhesus monkey kidney cells in the presence or absence of HBB. (From Eggers and Tamm, 1962a.)

There is substantial evidence that HBB has no effect on the rate of division of cells of several different types. Figure 10 demonstrates lack of effect of 219 μM HBB on the division of rhesus monkey kidney cells in primary culture *in vitro* (Eggers and Tamm, 1962a). The experiment lasted 60 hr during which the number of cells increased 2.5 times; reinforced Eagle's medium with 2% calf serum was used.

Figures 11 and 12 show that HBB also has no effect on division of HeLa and ERK cells in suitable media (Eggers and Tamm, 1961a). These cells divide more rapidly than monkey kidney cells. It is not known whether HBB would have an effect on cell division after the first 2 or 3 divisions in the continued presence of the drug.

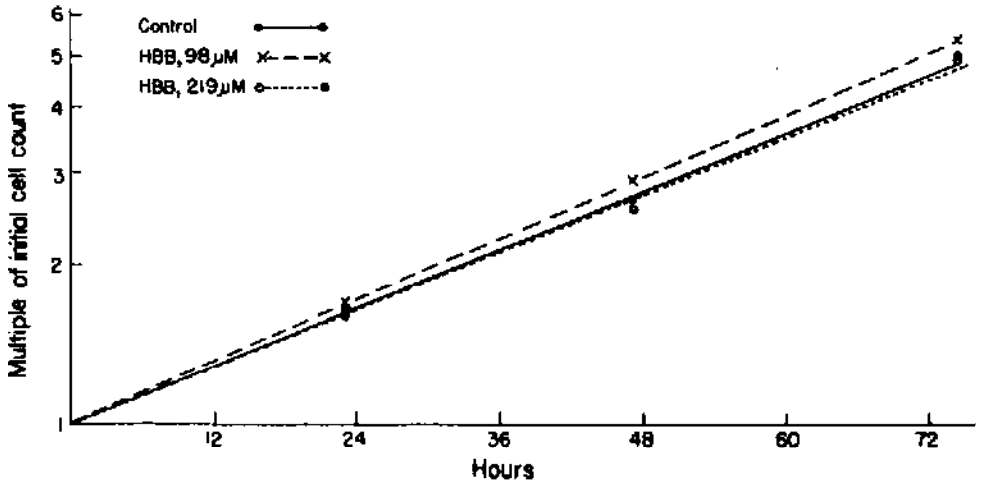


FIG. 11. Multiplication of HeLa cells in the presence or absence of HBB. (From Eggers and Tamm, 1961a.)

KINETICS OF ANTIVIRAL ACTION

The time course of the HBB-sensitive process in the virus replicative cycle has been established by exposing infected cells to the compound for varying periods during the growth cycle and measuring the effect on virus production. Figure 13 illustrates the kinetics of echovirus 12 multiplication in untreated monkey kidney cells and in cells treated with 219 μM HBB for 2.5, 4 or 12 hr after virus inoculation, following which the compound was removed (Eggers and Tamm, 1962a). Virus yields are recorded in hemagglutinating units. The latent period of echovirus 12 in the untreated cultures is 4 hr, followed by rapid increase in virus. Maximum virus yields are reached at 8–9 hr after inoculation. When cells are treated with

HBB for 2.5 hr or less following virus inoculation, the course of virus production is unaffected by the compound. Thus, no HBB-inhibitable process takes place during the first half of the latent period. When HBB is present for 4 hr and then removed, the latent period is extended to 5.5 hr. After removal of the compound, 1.5 hr are required before detectable amounts of new virus appear. This indicates that a process sensitive to HBB begins shortly after the midpoint of the latent period. In cells treated with HBB for 12 hr following infection, again 1.5 hr are required after removal of HBB before measurable amounts of virus are produced. It is apparent that the action of HBB is completely reversible: after removal of the compound, virus multiplies to full yields. However, as long as HBB is present, the drug-sensitive process cannot proceed.

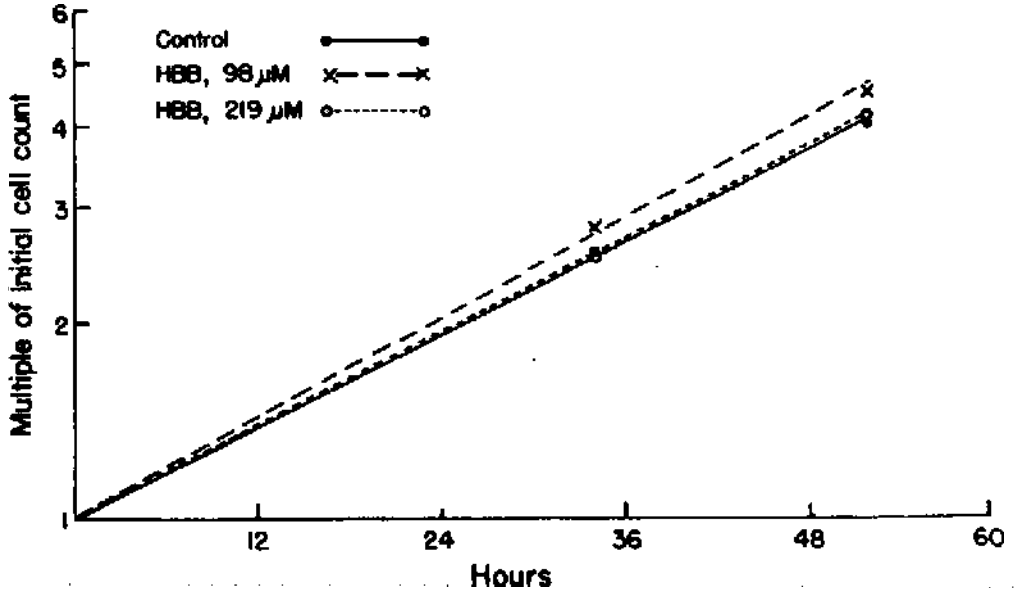


FIG. 12. Multiplication of ERK cells in the presence or absence of HBB. (From Eggers and Tamm, 1961a.)

Once the HBB-sensitive process has begun, HBB has a prompt inhibiting effect on virus production. Figure 14 illustrates the effects of HBB added at the end of the latent period or during the period of rapid increase in virus. The production of virus stops within 45 min after addition of compound. HBB also is effective when added as late as 5.5 hr after virus inoculation. That HBB is able to stop ongoing replication of echovirus 12 has also been demonstrated in single-cell studies (Caliguiri *et al.*, 1965).

These results indicate that the HBB-sensitive reaction is not one that takes place only during a brief period in the virus growth cycle. Rather, the drug-sensitive process begins during the second half of the latent period and continues during the rapid increase phase in virus replication. It is also evident that HBB is capable of causing essentially complete cessation of virus production.

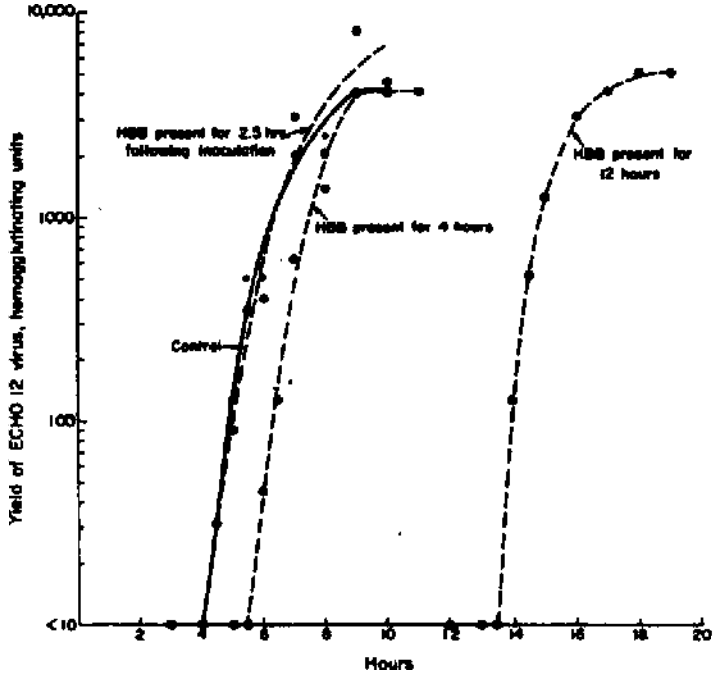


FIG. 13. Kinetics of echovirus 12 multiplication with 219 μM HBB present for 2.5, 4, or 12 hr following virus inoculation. Input multiplicity: 10–20 PFU/cell. (From Eggers and Tamm, 1962a.)

Similar results have been obtained with HBB and coxsackievirus A9 (Eggers *et al.*, 1963b). These will be discussed below, where the time course of the drug-sensitive process in the replication of the parent coxsackievirus A9 will be compared with that of the drug-requiring process in the replication of the drug-dependent variant of the virus.

It is possible that the effectiveness of HBB decreases during the final hours of virus production, as is the case with guanidine (Caliguiri and Tamm, 1968a, b); however, this has not been investigated with HBB. With guanidine, the drug-sensitive process terminates before maturation of all the progeny particles is completed.

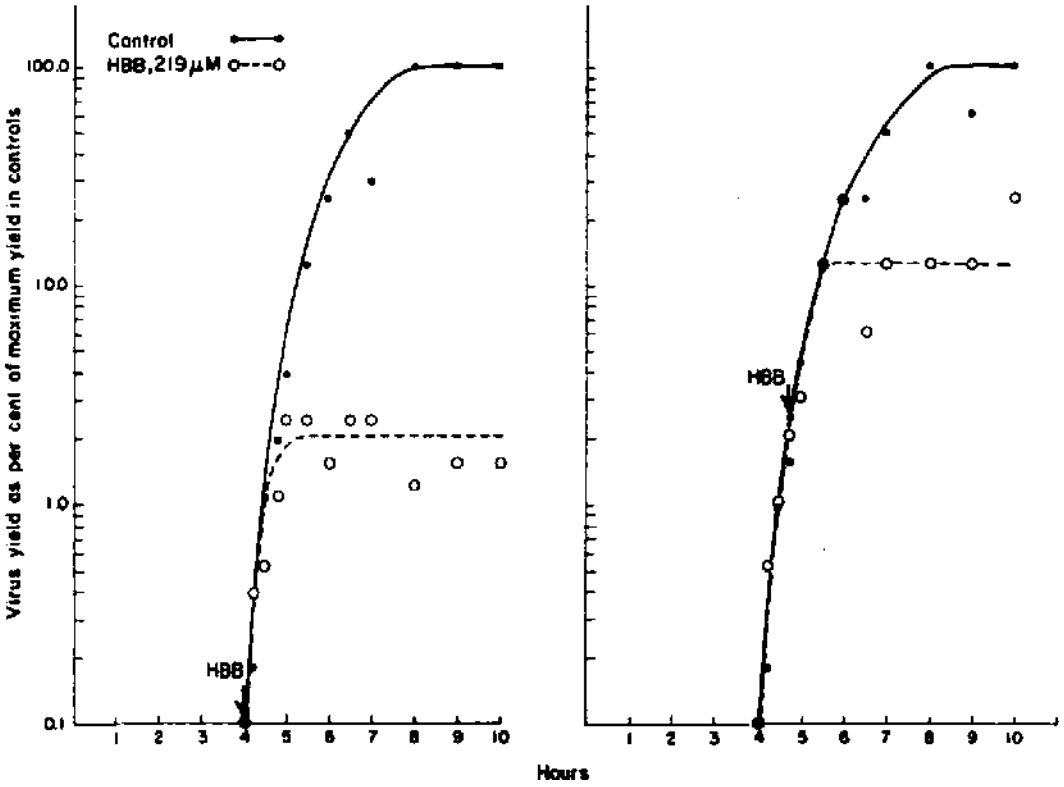


FIG. 14. Kinetics of echovirus 12 multiplication after addition of HBB at 4 or 4.75 hr in the viral growth cycle. Input multiplicity: 10–20 PFU/cell. (From Eggers and Tamm, 1962a.)

EFFECTS ON VIRUS BIOSYNTHESIS

The time course of the HBB-sensitive process in the multiplication of enteroviruses indicates that this compound does not affect the early processes of virus adsorption, penetration and uncoating (Eggers and Tamm, 1962a; Eggers *et al.*, 1963b; Eggers *et al.*, 1965). The available direct evidence also indicates that HBB has no effect on virus adsorption and penetration (Eggers and Tamm, 1962a). The central question therefore is as to what effects HBB has on the synthesis of virus RNA and proteins known to take place during the period of sensitivity to HBB in the virus growth cycle. Strong evidence has been obtained that HBB inhibits the replication of virus RNA (Eggers and Tamm, 1962a, 1963a), but does not have a direct effect on the synthesis of virus-directed proteins (Halperen *et al.*, 1964a, b). After inhibition of the synthesis of virus RNA, the synthesis of virus proteins, however, does diminish. The reduction in virus protein synthesis is probably a secondary consequence of inhibition

of synthesis of virus RNA, as the single-stranded virus RNA of picornaviruses functions as messenger in virus protein synthesis.

INHIBITION OF VIRUS RNA SYNTHESIS

Figure 15 illustrates the concentration dependence of the effect of HBB on the synthesis of the infective virus RNA of coxsackievirus A9. HBB (65 μM) causes marked but incomplete inhibition of the synthesis of virus RNA and of the production of virus particles (Eggers and Tamm, 1962a). In cells treated with 98 μM HBB no detectable amounts of new virus RNA or virus are made.

Inhibition of synthesis of coxsackievirus A9 RNA by HBB has also been demonstrated through measurements of the rate of incorporation of tritiated uridine into virus-specific RNA in actinomycin-treated cells (Fig. 16) (Eggers and Tamm, 1963a). Actinomycin D inhibits cell DNA-dependent RNA synthesis, but does not block virus RNA-dependent

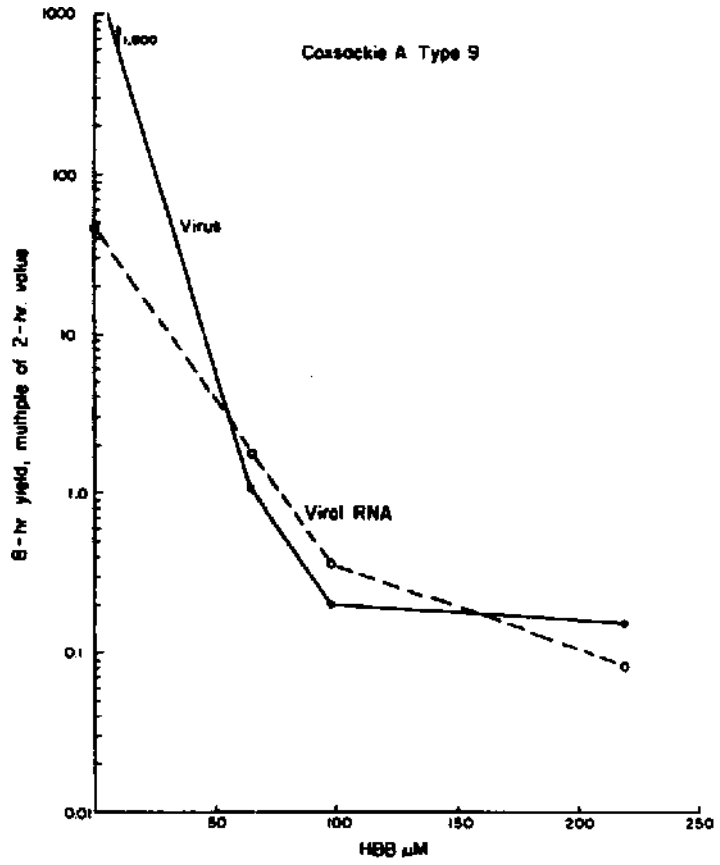


FIG. 15. Relationship between concentration of HBB and inhibition of synthesis of coxsackievirus A9 infective RNA and virus. (From Eggers and Tamm, 1962a.)

RNA synthesis (Reich *et al.*, 1961, 1962). Coxsackievirus A9-induced RNA synthesis becomes detectable between 3 and 4 hr after infection, and the rate of synthesis reaches a peak at 5 hr. Synthesis of virus-specific RNA then decreases and is no longer detectable at 8 hr. Figure 16 shows that 219 μM HBB completely prevents the virus-induced incorporation of tritiated uridine into RNA.

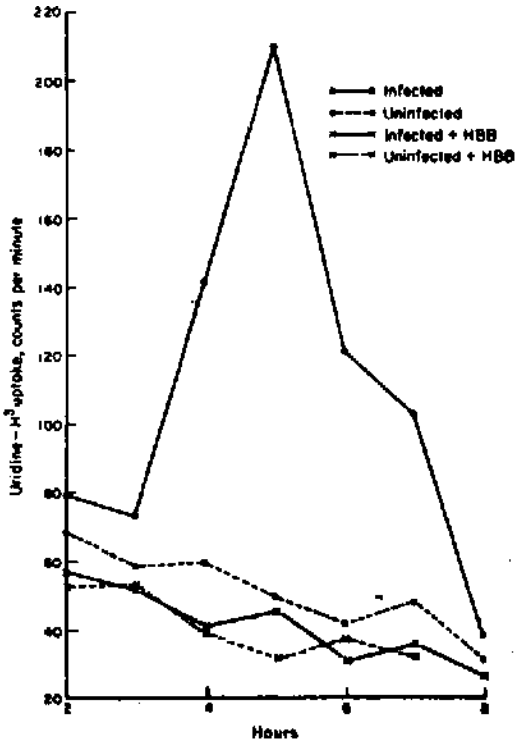


FIG. 16. Coxsackievirus A9 RNA synthesis in actinomycin-treated monkey kidney cells, and its inhibition by 219 μM HBB. Incorporation of tritiated uridine into acid-insoluble form was determined after a 15-min pulse. ●——●, infected; ● - - - ●, uninfected; ×——×, infected + HBB; × - - - ×, uninfected + HBB. (From Eggers and Tamm, 1963a.)

With echovirus 12, as with coxsackievirus A9, 98 μM HBB completely inhibits the synthesis of infective virus RNA and virus (Fig. 17) (Eggers and Tamm, 1962a). Although 65 μM HBB is insufficient to inhibit completely the synthesis of infective virus RNA, no new virus could be detected in untreated cultures 8 hr after infection. There is evidence that this dichotomy is probably not due to a differential effect on virus RNA synthesis and virus production, but merely reflects emergence of drug-resistant virus RNA.

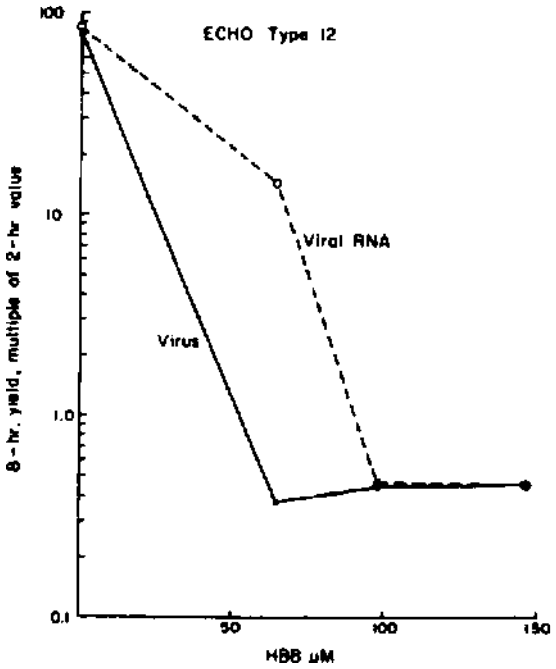


FIG. 17. Relationship between concentration of HBB and inhibition of synthesis of echo 12 infective virus RNA and virus. (From Eggers and Tamm, 1962a.)

Figure 18 shows that 65 μM HBB delays the onset of synthesis of virus RNA, but once synthesis has begun, it proceeds at a rapid rate. The production of virus particles is also delayed. The important fact is that when virus production does begin, the virus produced is demonstrably resistant to HBB (Halperen, Eggers and Tamm, 1963, unpublished work). Thus, the lower HBB concentration permits early emergence of a population of resistant echovirus 12 particles.

HBB prevents the appearance in poliovirus-infected HeLa cells of virus RNA polymerase activity in detectable amounts (Baltimore *et al.*, 1963). When the inhibitor is added to infected cells during the exponential increase phase in virus multiplication, it causes a marked decrease in the level of polymerase activity demonstrable by assay of cell extracts. However, HBB has no demonstrable effect on the activity of the crude enzyme preparation when it is added to the cell-free *in vitro* assay system for polymerase activity. Such a preparation is an aggregate of template and enzyme; while RNA chains can be completed in the *in vitro* system, few or no new chains may be initiated. The initial interpretation of the findings with HBB was that HBB might interfere with the messenger function of virus RNA in the synthesis of virus RNA polymerase (Baltimore *et al.*,

1963). In view of a number of recent findings, this interpretation no longer appears valid (Caliguiri and Tamm, 1968a, b). It is probable that HBB, like guanidine, blocks the initiation of new chains of virus RNA.

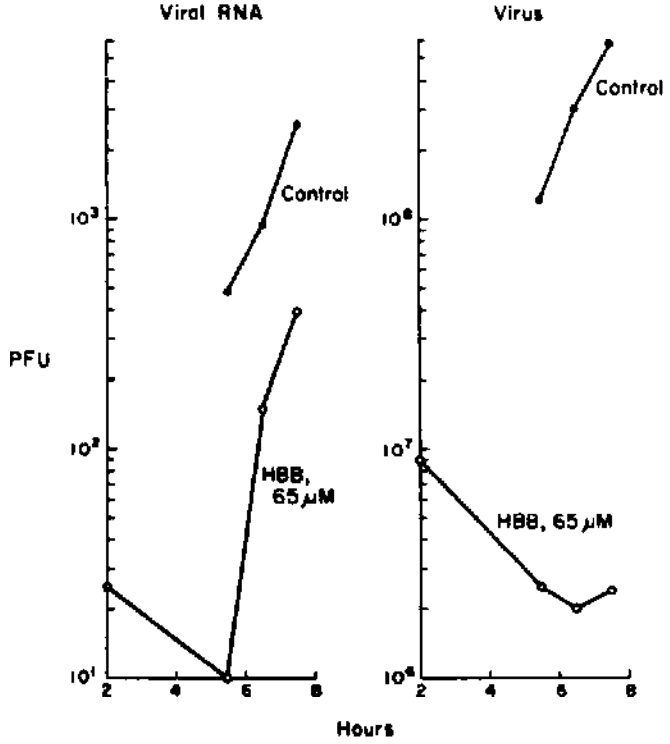


FIG. 18. Effects of 65 μM HBB on the kinetics of synthesis of echovirus 12 infective RNA and virus. (From Eggers and Tamm, 1962a.)

LACK OF DIRECT EFFECT ON VIRUS PROTEIN SYNTHESIS

HBB has no direct effect on the formation of echovirus 12 capsid proteins (Halperen *et al.*, 1964b). The findings on which this conclusion is based may be summarized as follows: in the course of echovirus 12 multiplication two kinds of virus particles are made: complete infective virions and empty capsids. Despite the arrest by HBB of synthesis of virus RNA and complete virus particles in echovirus 12-infected cells, synthesis of empty capsids continues for a time. Both empty and complete virus particles agglutinate erythrocytes, which permits ready biological assay. If echovirus-infected cells are treated in sequence with *p*-fluorophenylalanine and HBB, considerable quantities of empty capsids are made without production of any infective virions. The quantity of empty capsids made depends on the length of preliminary incubation with *p*-fluorophenylalanine. Both virus RNA polymerase and virus RNA can be synthesized in the presence of this amino-acid analog (Eggers *et al.*, 1963a), but capsid proteins made in the presence of the analog are apparently defective and

fail to be assembled into either complete or empty capsids. The results of sequential treatment of echovirus 12-infected cells with *p*-fluorophenylalanine and HBB indicate that virus RNA synthesized in the presence of *p*-fluorophenylalanine is able to serve as messenger in the synthesis of

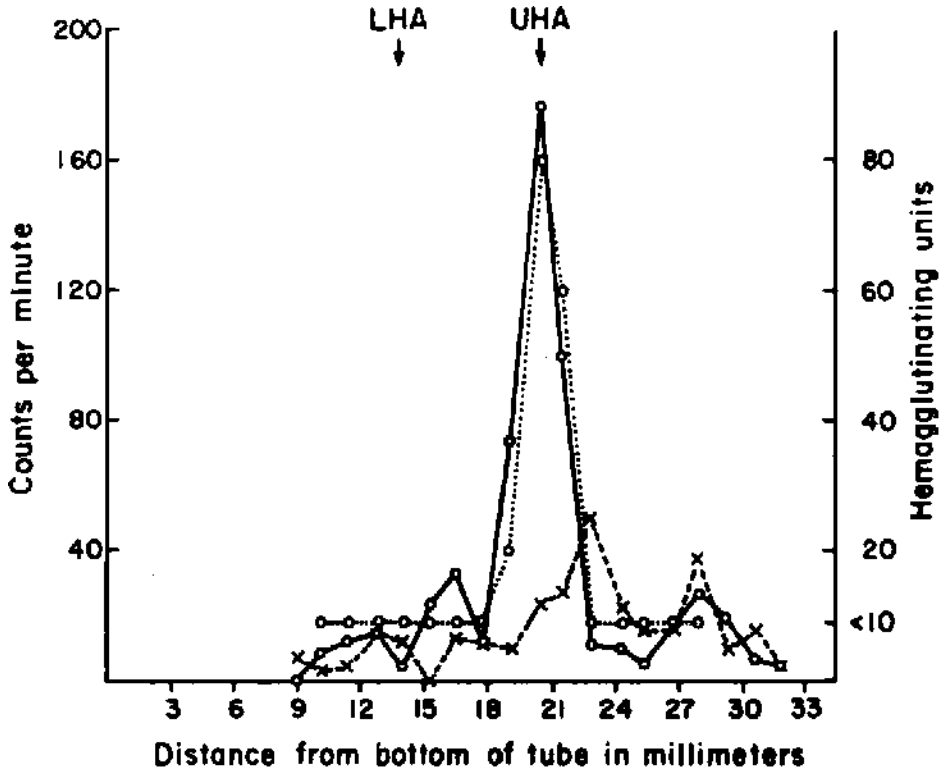


FIG. 19. Synthesis of echovirus 12 capsid proteins and formation of empty hemagglutinating capsids in the presence of HBB. LHA, lower hemagglutinin, density 1.29; UHA, upper hemagglutinin, density 1.33. Echovirus 12-infected monkey kidney cells were treated with 3 mM *p*-fluorophenylalanine for 7 hr, and with 219 μM HBB for 3 hr. During the first 2 hr of treatment with HBB, the medium contained tritiated DL-leucine and DL-lysine. Cell extracts were treated with ether and Genetron 113 and analyzed by density gradient in CsCl. Fractions were divided into two aliquots, one of which was used for hemagglutination titration (o.... o), and the other was absorbed with erythrocytes, which were collected and counted in a windowless gas flow counter (o——o). Uninfected cells were treated identically and served as controls (× – – – – ×). (From Halperen *et al.*, 1964b.)

functional capsid proteins after *p*-fluorophenylalanine has been replaced in the medium with HBB (Fig. 19) (Halperen *et al.*, 1964b). The capsid proteins newly synthesized in the presence of HBB can assemble into empty capsids. Thus, HBB has no detectable effect on the messenger

function of virus RNA in the synthesis of virus capsid proteins.

There is also evidence that HBB does not prevent poliovirus-induced inhibition of cell biosynthesis (Bablanian and Tamm, 1963, unpublished work), which is thought to be mediated through newly synthesized virus-specific proteins.

MECHANISM OF ACTION

The results with guanidine (cf. following chapter) have in all respects been similar to those obtained with HBB. It now appears that there is a mechanism other than inhibition of synthesis of virus RNA polymerase which would satisfactorily explain the reduction in virus RNA polymerase activity observed after treatment of infected cells with HBB or guanidine (Caliguiri and Tamm, 1968a). Results obtained with guanidine indicate that the inhibitor has an effect on the initiation of virus RNA chains. If guanidine and HBB indeed interfere with chain initiation, virus RNA polymerase in infected cells would be expected to become deficient in or devoid of RNA template soon after treatment with inhibitor is begun. Failure of reassociation with template RNA molecules in drug-treated cells could manifest itself as decreased RNA polymerase activity of the enzyme assayed *in vitro*. The fact that HBB and guanidine have no effect on virus RNA synthesis when added to the *in vitro* assay system for virus RNA polymerase (Baltimore *et al.*, 1963) may be taken as evidence that the inhibitors have no effect on the growth of previously initiated RNA chains. Very little, if any, chain initiation is likely to take place in aggregate enzyme preparations assayed *in vitro*.

The question may be raised as to whether HBB has any direct effect on the assembly of complete virus particles, although it clearly does not interfere with formation of empty capsids. The likeliest assumption is that the effect of HBB on production of infective virus particles is entirely secondary to rapid inhibition of virus RNA synthesis. A direct effect on the assembly process would be detectable only in a system in which assembly of virus takes place from preformed virus precursors. In the absence of such a system, no findings have been obtained to provide a conclusive answer to this question (Caliguiri and Tamm, 1968b).

Although all of the available evidence indicates that HBB and guanidine inhibit the same virus-specific process, i.e. the synthesis of virus RNA, there is reason to believe that the primary sites of action of HBB and guanidine are not identical (*vide infra*).

EFFECTS ON VIRUS-INDUCED ALTERATIONS IN CELLS

HBB markedly delays the development of virus-induced morphological changes in monkey kidney cells infected with enteroviruses, such as echovirus 12 or coxsackievirus B4 (Bablanian *et al.*, 1966). The compound does not prevent the ultimate degeneration of cells, even though the replication of virus RNA and production of virus are inhibited. The mechanism responsible for the development of delayed virus cytopathic effects in HBB-treated cells is not clear. There are two main possibilities: (1) infection with echovirus 12 or coxsackievirus B4 may cause early inhibition of cell biosynthesis, and the late morphological changes may be a secondary consequence of such inhibition; (2) the virus-induced morphological changes may occur independently of any virus-induced inhibition of cell biosynthesis.

As to the first possibility, a pertinent finding is that HBB does not prevent poliovirus-induced early inhibition of cell RNA and protein synthesis (Bablanian and Tamm, 1963, unpublished work). Whether echovirus 12 and coxsackievirus B4 in fact cause such early and marked inhibition of cell biosynthesis in primary cultures of rhesus monkey kidney cells is not known. Technically this would be difficult to determine, as even at very high input multiplicities only a proportion of cells becomes infected. In LLC-MK2 cells, a continuous line of rhesus monkey kidney cells, echovirus 12 infection does lead to a decrease in the number of electron microscopically identifiable polyribosomes (Skinner *et al.*, 1968). As HBB does not prevent this decrease, it is quite likely that cell protein synthesis becomes inhibited in HBB-treated infected cells.

It is also possible, however, that the virus-induced morphological changes which develop late in HBB-treated cells occur independently of virus-induced inhibition of cell biosynthesis. Virus RNA can function as messenger in the synthesis of virus capsid proteins even in the presence of HBB (Halperen *et al.*, 1964b). It may be that a virus-specific protein, which may in fact be a capsid protein, is slowly synthesized in the presence of HBB and ultimately induces morphological changes in the absence of replication of the virus genetic material. Such a cytotoxic virus protein may act by causing release of lysosomal enzymes, by altering the plasma membrane, or through some other mechanism.

The effects of HBB on enterovirus-induced alterations in cells (Skinner *et al.*, 1968) are in all regards quite similar to those previously observed with guanidine and poliovirus (Bablanian *et al.*, 1965a, b; Dales *et al.*, 1965). HBB prevents the development in echovirus 12-infected LLC-MK2

cells of cytoplasmic membrane-bounded bodies (Skinner *et al.*, 1968), a finding also obtained with guanidine in poliovirus-infected HeLa cells (Dales *et al.*, 1965). The membrane-bounded bodies develop by a mechanism comparable to that of the formation of autolytic vacuoles, and constitute islets of segregated cytoplasmic matrix. They are unlike vacuoles normally present. Even though some of these bodies contain progeny virus, the association between membrane-bounded bodies and virus is not obligatory. The formation of the membrane-bounded bodies appears to represent a response of the cell to infection and may take extreme forms, such as in poliovirus infection.

In echovirus 12 infection of LLC-MK2 cells, cytoplasmic membrane-bounded bodies are first seen by electron microscopy 4–5 hr after infection and increase in number with time (Skinner *et al.*, 1968). Cytopathic changes, such as rounding of cells observed by phase contrast microscopy, develop 5–6 hr after infection. The addition of HBB to cultures at the time of infection or during the first half of the latent period prevents the development of membrane-bounded bodies as well as retraction and rounding in over 95% of the cells during a 7-hr period. However, if HBB is not added until 3 or 4 hr after infection, the development of membrane-bounded bodies and cell retraction are not prevented.

Kinetic studies of the time course of the HBB-inhibitable process in echovirus 12 replication have shown that it begins 2 hr after infection (Eggers and Tamm, 1962a). Thus, when HBB is added 3 or 4 hr after infection, some biosynthesis of virus RNA and capsid proteins has already taken place. HBB stops further synthesis of virus RNA, but permits continued synthesis, for a time, of virus proteins (Halperen *et al.*, 1964b). Clearly, the time in the virus growth cycle after which HBB no longer prevents the virus-induced formation of cytoplasmic membrane-bounded bodies corresponds to the period of active synthesis of virus RNA and proteins. Production of new virus particles in detectable amounts follows within about 2 hr.

The evidence obtained with echovirus 12 indicates that formation of the membrane-bounded bodies, demonstrable by electron microscopy, occurs under the same conditions as development of pathological changes in cell shape, revealed by light microscopy. The development of both types of cytopathological alterations depends on virus biosynthesis. This relationship between development of cytopathic changes and virus biosynthesis corresponds closely to that established for poliovirus in human embryonic lung cells (HEL cells) with the aid of guanidine and puromycin (Bablanian *et al.*, 1965a, b).

DRUG RESISTANCE AND DRUG DEPENDENCE

DRUG RESISTANCE

When cells infected with a drug-sensitive virus are treated with HBB over a period of days, resistant populations commonly emerge. This is illustrated in Fig. 20. The parent coxsackievirus A9 is highly sensitive to HBB. Virus which emerged in the presence of HBB during two passages is resistant to inhibition by this compound (Eggers and Tamm, 1961a; Tamm and Eggers, 1963b). Experiments with HBB at very high concentrations have shown that the variant virus is not completely resistant to HBB; 493 μM HBB does delay, although it does not prevent, the development of its cytopathic effects, which indicates that the multiplication of the resistant particles is still partially inhibited. Comparison of the responses to HBB of clonal populations of sensitive and resistant virus particles has given confirmatory results. HBB-sensitive particles are unable to produce plaques in the presence of 98 μM HBB, while the resistant particles are able to do so. However, the reduced size and number of plaques produced by resistant virus in the presence of HBB indicates again that the variant particles are not completely resistant.

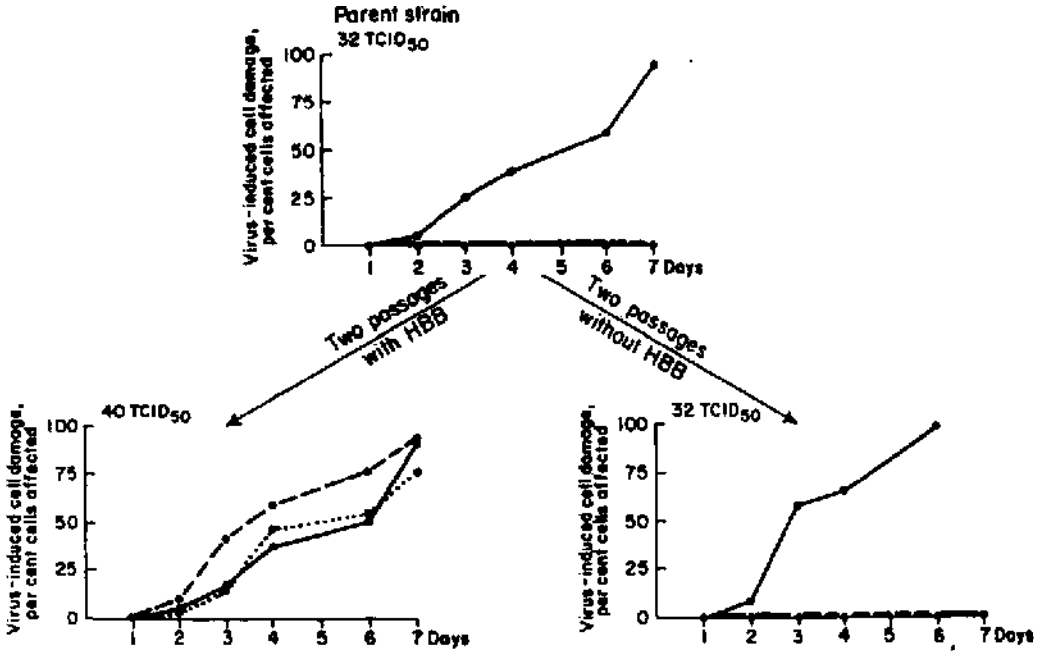


FIG. 20. Emergence of an HBB-insusceptible variant of coxsackievirus A9 after two passages in the presence of 98 μM HBB. Concentration of HBB: —— none, - - - - 98 μM, ... 219 μM. (From Eggers and Tamm, 1961a.)

Clonal populations of sensitive and resistant virus have been passaged five times without changes in sensitivity or resistance to HBB, suggesting that, in the absence of HBB, the genetic character concerned with drug sensitivity or resistance is not highly unstable.

The rapidity with which a drug-resistant variant of an originally sensitive virus appears varies from one virus to another, and depends on the amounts of virus and HBB employed. Resistant populations emerge sooner with relatively large inocula of virus and low concentrations of inhibitor. With HBB, resistant populations generally emerge sooner with polioviruses than with coxsackie B or echoviruses. A variant which has emerged in infected cultures treated with HBB and is markedly resistant to this compound may not be equally resistant to guanidine, and vice versa (Tamm and Eggers, 1962). Combined treatment with both HBB and guanidine has a synergic effect, and a suppressive effect on the emergence of resistant variants (see below).

Critical experiments have not yet been done to determine whether the resistant mutants appear spontaneously or are drug-induced. The first mechanism is the classical one in mutation of microorganisms to drug resistance, and may well be involved in mutation of picornaviruses to altered sensitivity to HBB. However, available evidence suggests that HBB may also perhaps induce or facilitate mutation to drug resistance (Tamm and Eggers, 1963a). Often a resistant population emerges when fewer than 100 infective units of sensitive wild-type parent virus are inoculated per culture. Plating experiments with parent populations have shown that if they contain any resistant particles at all they are present in a proportion smaller than 1 in 10,000. Thus, the possibility exists that HBB acts not only as a selective agent, but is responsible for the initial appearance of drug-resistant particles. In contrast to many mutagens, such as methylating agents or x-rays, HBB, even at high concentration, has no direct inactivating effect on virus particles (Eggers and Tamm, 1961a; Tamm *et al.*, 1961) or infective virus RNA (Eggers and Tamm, 1962a). If HBB induces mutations by acting directly on virus RNA, its effects are probably restricted and specific. There is also the possibility that HBB does not act directly on virus RNA, but that it increases the probability of spontaneous mutations by extending the time during which virus RNA may undergo mutations or by creating conditions in the infected cells that may facilitate such mutations. When HBB-treated infected cells are incubated for several days, the RNA of a sensitive virus is ready to replicate, but is prevented from replicating or is replicating at a very slow rate. Clearly, this may increase the probability of mutation in the virus

RNA molecules. An important experiment would be to determine whether, under these conditions, mutations in properties other than those having to do with drug sensitivity are also taking place at high rates.

DRUG DEPENDENCE

In addition to resistant mutants, HBB-dependent mutants of enteroviruses have also been isolated (Eggers and Tamm, 1962b, 1963c). This represents the first demonstration of drug dependence in viruses. Dependent mutants of coxsackievirus A9 and echovirus 13 emerged during

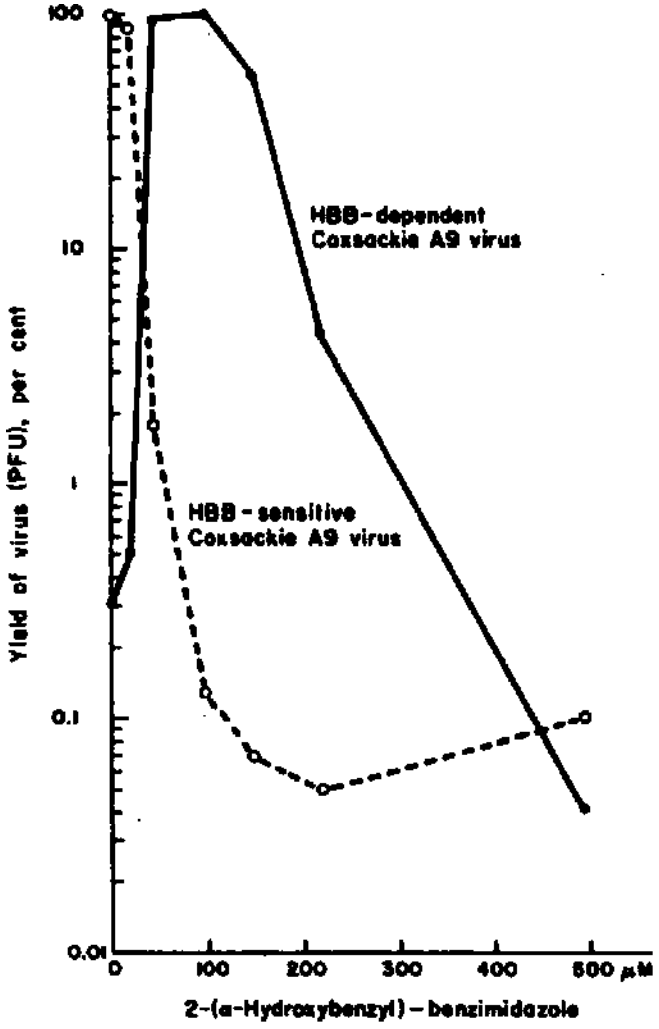


FIG. 21. Effects of HBB on yields of HBB-dependent or HBB-sensitive coxsackievirus A9. The maximum yield of dependent virus in the presence of HBB was 1.2×10^8 PFU/ml, and that of sensitive virus in the absence of HBB was 1.6×10^8 PFU/ml. (From Eggers and Tamm, 1963c.)

attempts to obtain resistant variants by infecting cells with sensitive parent strains in the presence of HBB.

HBB-dependent virus can adsorb and penetrate in the absence of the compound (Eggers and Tamm, 1963c). HBB dependence is inherent in the genetic material of the virus and involves the expression of the reproductive potentialities of the virus RNA. Infective RNA extracted from HBB-dependent coxsackievirus A9 is itself also HBB-dependent; it is unable to direct production of detectable amounts of dependent virus in the absence of compound, but is able to do so in its presence (Eggers and Tamm, 1962b, 1963c).

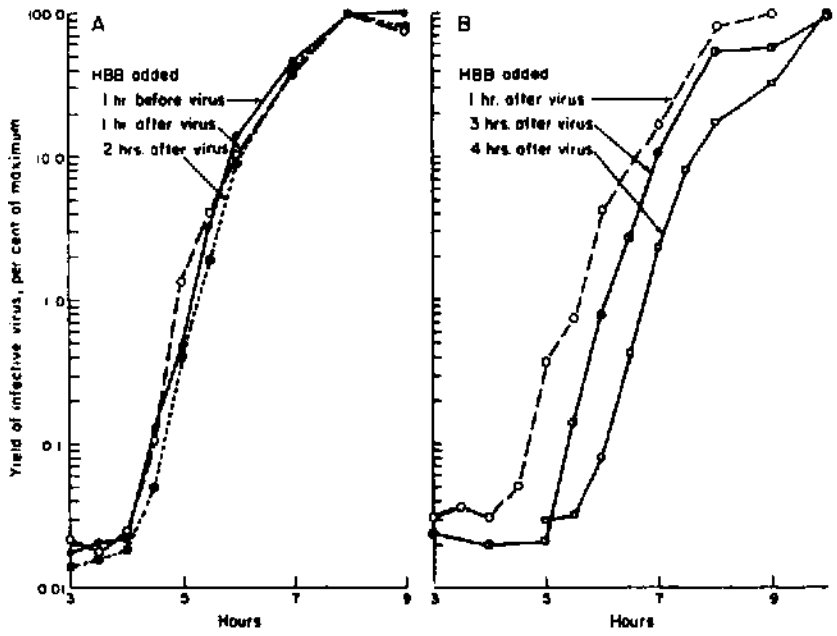


FIG. 22. Kinetics of multiplication of HBB-dependent coxsackievirus A9 when 98 μM HBB was added 1, 2, 3, or 4 hours following virus inoculation. (From Eggers *et al.*, 1963b.)

HBB is required for the synthesis of HBB-dependent virus RNA. This has been demonstrated with HBB-dependent coxsackievirus A9 by measuring the synthesis of HBB-dependent infective virus RNA and by determining the incorporation of uridine-^{3}H into virus RNA in actinomycin-treated cells (Eggers *et al.*, 1963b).

The available evidence suggests that dependence and sensitivity may involve opposite drug effects at the same site of action; that is, the drug-dependent and the drug-sensitive processes appear to be biochemically

analogous (Eggers *et al.*, 1963b). In a variety of experiments, dependence and sensitivity manifest themselves as the precise opposites of one another. Figure 21 shows that there is a striking similarity in concentrations of HBB required for maximum growth of HBB-dependent virus or for marked inhibition of growth of the HBB-sensitive parent virus (Eggers and Tamm, 1963c).

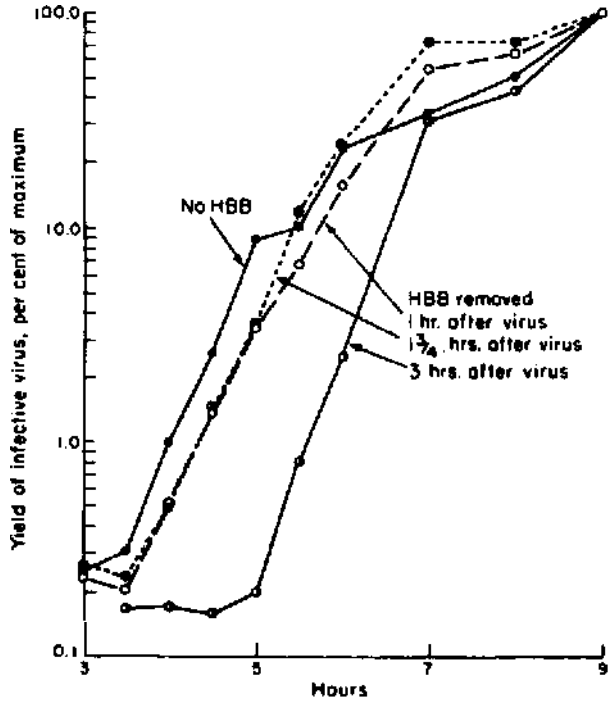


FIG. 23. Kinetics of multiplication of HBB-sensitive coxsackievirus A9 with HBB (219 μM) present for 1, 1¾ or 3 hr following virus inoculation. (From Eggers *et al.*, 1963b.)

Drug-dependent virus, in the presence of HBB at optimal concentration, replicates with kinetics which are similar to those that characterize the growth of the drug-sensitive parent virus in the absence of the compound (Eggers and Tamm, 1963c). In addition, as illustrated in Figs. 22, 23 and 24, 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 (Eggers *et al.*, 1963b). The outstanding kinetic features are that HBB is not required during the first half of the latent period in the replication cycle of the dependent virus, but is required during the second half (Fig. 22), and also during the phase of exponential increase in virus (Fig. 24). Correspondingly

HBB is not inhibitory for the sensitive virus during the first half of the latent period, but is inhibitory during the second half (Fig. 23), and also during the rapid increase phase in virus growth (Fig. 24). All the evidence is compatible with the view that HBB enables the drug-dependent mutant virus to carry out a step in virus RNA synthesis, which the compound inhibits during the synthesis of the RNA of drug-sensitive wild type virus.

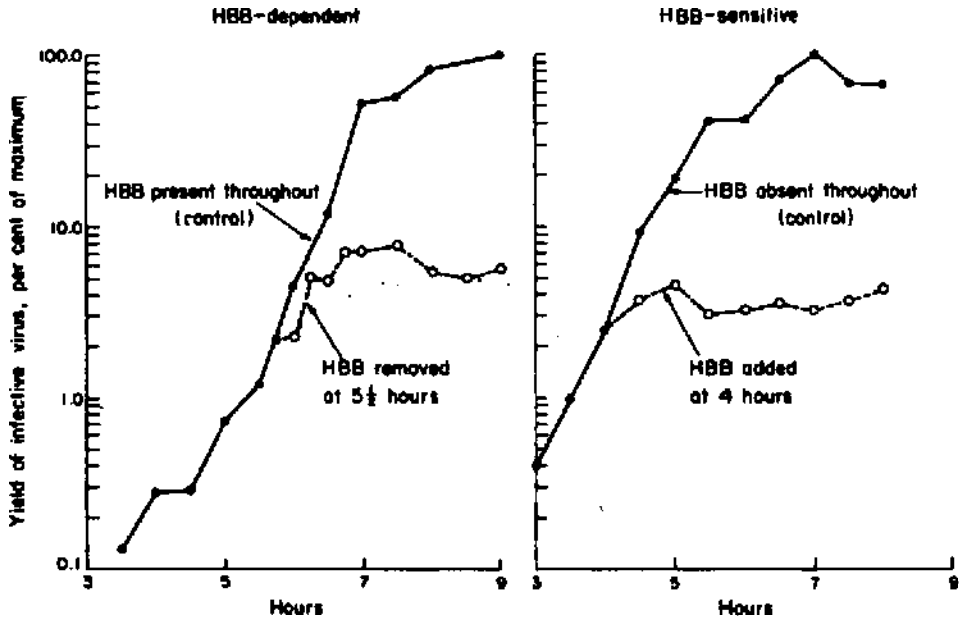


FIG. 24. Kinetics of multiplication of HBB-dependent coxsackievirus A9 after removal of HBB, and of HBB-sensitive virus after addition of HBB during the exponential increase phase in virus growth. HBB-dependent virus was grown in the presence of 98 μM HBB; 219 μM HBB was used to inhibit the sensitive virus. (From Eggers *et al.*, 1963b.)

Since we may assume that there are structural differences between the mutant and wild type viruses in the RNA and in at least one of the proteins, the structural requirements of enhancement and inhibition would not necessarily be the same. The requirements for selective inhibitory activity are in fact more stringent than those for enhancing activity. As can be seen in Table 10, maximal growth of the HBB-dependent mutant of coxsackievirus A9 can also be obtained with 2-(*o*-hydroxybenzyl)-benzimidazole and 5-methyl-2-D-ribobenzimidazole, as well as with unsubstituted benzimidazole. However, 2-(*o*-hydroxybenzyl)benzimidazole

possesses only slight virus-inhibitory activity and 5-methyl-2-D-ribobenzimidazole and unsubstituted benzimidazole lack selective virus-inhibitory activity. With another derivative which lacks selective inhibitory activity, i.e. 2-ethyl-5-methylbenzimidazole, only near-toxic concentrations give some growth of dependent virus (Eggers and Tamm, 1963c). Imidazole, adenosine, arginine, histidine, and creatinine are ineffective in supporting the growth of drug-dependent virus.

TABLE 10. EFFECTS OF BENZIMIDAZOLE DERIVATIVES ON PLAQUE FORMATION BY HBB-DEPENDENT OR HBB-SENSITIVE COXSACKIEVIRUS A9

Benzimidazole derivative	Concentration (μM)	Infectivity titer (PFU)	
		HBB-dependent virus, % increase (relative to HBB)	HBB-sensitive virus, % inhibition (relative to HBB)
2-(α-Hydroxybenzyl) (HBB) (reference compound)	98	100[a]	100[b]
2-(*o*-Hydroxybenzyl)	98	52	0.1
	219	120	14
5-Methyl-2-D-ribobenzimidazole-unsubstituted	3500	71	11
	50	0	
	250	0.8	
	1000	100	0
	3000	56	4.8
2-Ethyl-5-methyl	50	0	0
	150	0.4	0
	300	0.6	0
	600	7.5	0.1

[a] HBB increased infectivity titer 427-fold: from 4.57×10^5 in its absence to 1.95×10^8 PFU/ml in its presence.

[b] HBB reduced the infectivity titer 4080-fold: from 3.16×10^8 in its absence to 7.75×10^4 PFU/ml in its presence. From Eggers and Tamm, 1963c.

Guanidine·HCl, a selective inhibitor of enterovirus multiplication, fully supports the growth of HBB-dependent enteroviruses (Eggers and Tamm, 1962b, 1963c). Figure 25 shows that the concentration of guanidine

required for maximal enhancement of dependent virus is similar to that which causes marked inhibition of sensitive wild-type virus (Eggers and Tamm, 1963c). The guanidine-requiring process also begins during the second half of the latent period (Eggers *et al.*, 1965b). Determination of the duration of the guanidine-requiring process in the growth cycle is

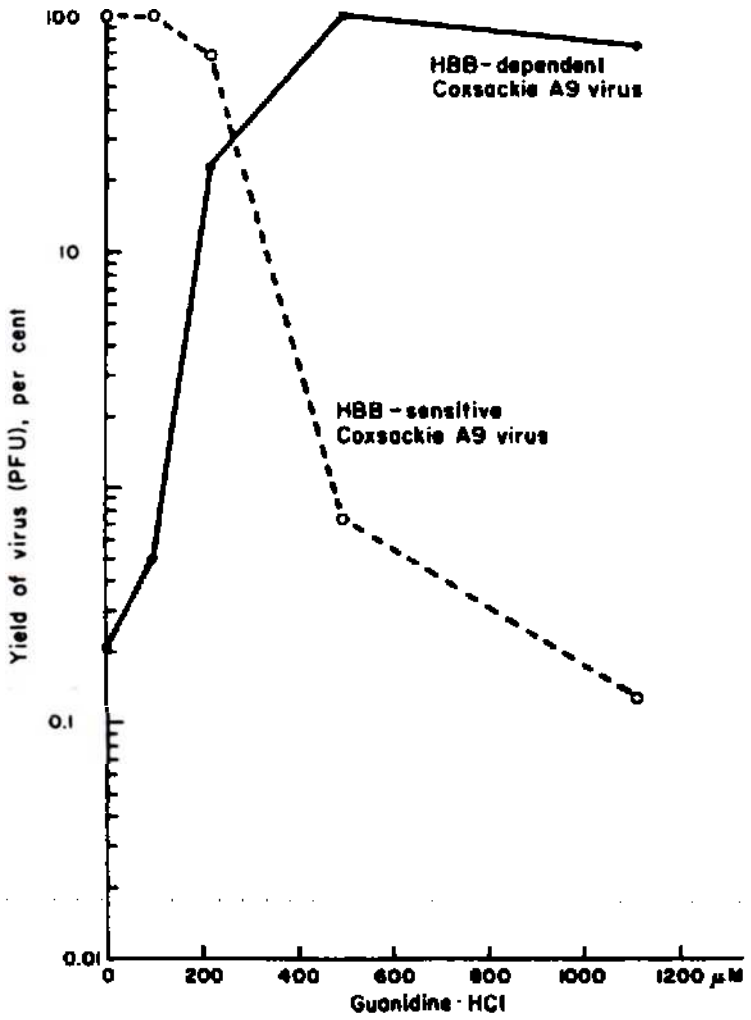


FIG. 25. Effects of guanidine on yields of HBB-dependent or HBB-sensitive coxsackievirus A9. The maximum yield of dependent virus in the presence of guanidine was 1.6×10^8 PFU/ml, and that of sensitive virus in the absence of HBB was 1.6×10^5 PFU/ml. (From Eggers and Tamm, 1963c.)

complicated by the fact that guanidine cannot be readily removed from monkey kidney cells by washing.

Quantitatively, the drug requirements of different dependent variants are not identical. For example, the HBB-dependent variant of echovirus 13

requires somewhat higher concentrations of HBB or guanidine for maximum enhancement than does HBB-dependent coxsackievirus A9 (Eggers and Tamm, 1963c).

The precise mechanisms are not known by which HBB and guanidine enable the replication of the RNA of drug-dependent viruses. Based on all the available evidence the hypothesis has recently been proposed that guanidine inhibits chain initiation in the replication of the RNA of sensitive picornaviruses (Caliguiri and Tamm, 1968a). Growth and completion of virus RNA chains do not appear to be inhibited by guanidine. The primary site and precise biochemical mechanism of action of guanidine have not yet been determined. A possible target molecule is the virus RNA polymerase. Similar considerations may be entertained for HBB, whose biochemical action is closely similar to that of guanidine. The differences in the virus-inhibitory spectrum and other aspects of action which have been noted may be related to genetic fine structure of the target molecules, and not to principal differences in the primary sites of action of HBB and guanidine. The simplest hypothesis to explain drug dependence is that the target molecule is the same as that involved in sensitivity, but altered in its configuration and functional properties through a mutational step.

GENETIC STUDIES

MUTATION FROM HBB DEPENDENCE TO INDEPENDENCE

As has been mentioned above, passage of HBB-sensitive wild-type virus in the presence of HBB commonly leads to emergence of HBB-resistant mutants of varying degrees of resistance (Eggers and Tamm, 1961a; Tamm and Eggers, 1963b). Occasionally, HBB-dependent mutants emerge (Eggers and Tamm, 1962b, 1963c). The simplest explanation of these findings is to assume that, during replication of HBB-sensitive virus in the presence or absence of HBB, mutants resistant to or requiring HBB arise spontaneously and are selected during passage of virus in the presence of HBB. The alternate possibility that HBB may itself induce or facilitate mutation of drug-sensitive virus to drug resistance or drug dependence was also discussed in a preceding section.

The rates of mutation from HBB sensitivity to resistance or dependence have not been determined; however, some information is available concerning mutation from drug dependence to drug independence. That drug-independent mutants arise with considerable frequency during

replication of HBB-dependent virus in the presence of HBB was recognized early (Eggers and Tamm, 1963c). Even after repeated cloning, the HBB-dependent coxsackie A9 variant gives rise to populations containing 0.2–1% of HBB-independent particles. Back mutation is either to drug resistance or sensitivity. The fact that back mutation to resistance is observed more commonly may be due to the conditions of the experiments, namely the presence of HBB, which probably selectively favors the multiplication of drug-resistant revertants by inhibiting the multiplication of drug-sensitive revertants. This situation has subsequently been circumvented by the use of unsubstituted benzimidazole, which permits optimal multiplication of HBB-dependent coxsackievirus A9 at concentrations which do not inhibit the replication of sensitive particles (Eggers and Tamm, 1965). In these studies, HBB-dependent virus was plated in the presence of 1 μM unsubstituted benzimidazole and three plaques which developed at high dilution were picked. The amount of HBB-dependent or independent virus present in each plaque was determined by titration in the presence or absence of 100 μM HBB. The mutation indices thus derived indicate a mutation frequency from dependence to independence of the order of 10^{-4} mutations per replication.

The HBB-independent revertants show varying degrees of sensitivity or resistance (Eggers and Tamm, 1965). To isolate the revertants, virus from the three plaques initiated by dependent virus was plated in the absence of the compound. The developing plaques were picked at random and subjected to one passage in monkey kidney tube cultures in the absence of the compound to eliminate residual HBB-dependent virus which may have contaminated the drug-independent clones. A total of 79 clones of drug-independent virus derived from drug-dependent virus were thus prepared. Table 11 shows that the 79 drug-independent clones were distributed over a wide range of sensitivity or resistance to HBB. The grouping in Table 11 is arbitrary; each group is composed of clones exhibiting many varying degrees of sensitivity or resistance, so that there is a more or less continuous series of variants. At one extreme, the most sensitive clone showed a ratio of the number of plaques formed in the absence of the compound to the number formed in its presence equal to 1.7×10^5. At the other extreme some clones gave ratios of 1.

The genetic stability of the revertant virus particles has been investigated by determining the sensitivity or resistance of subclones derived from 5 clones grouped either as intermediate or resistant (Eggers and Tamm, 1965). Without exception all subclones exhibited the same response as the parent clones. Thus, HBB-independent revertant particles are not highly

unstable genetically. It is therefore highly probable that the HBB-independent clones examined were, in the great majority cases, derived directly from HBB-dependent virus, and did not represent the end result of two or more mutational events.

TABLE 11. SENSITIVITY TO 2-(α-HYDROXYBENZYL)BENZIMIDAZOLE (HBB) OF HBB-INDEPENDENT MUTANT CLONES DERIVED FROM THREE HBB-DEPENDENT PARENT CLONES OF COXSACKIEVIRUS A9

Parent clones (HBB-dependent)	No. of mutant clones[a] (HBB-independent)		
	Sensitive	Intermediate	Resistant
Plaque 1	4	7	9
Plaque 2	11	9	12
Plaque 3	10	10	8
Total	25	26	28

[a] HBB sensitivity is expressed as the ratio of the number of plaque-forming units (PFU) obtained without HBB in the overlay to the number of PFU with 100 μM HBB in the overlay. Sensitive clones, ratios $\geq 4 \times 10^3$; intermediate clones, ratios from 3.9×10^3 to 2; resistant clones, ratios from 1.9 to 1.

From Eggers and Tamm, 1965.

RESCUE

Guanidine-sensitive or guanidine-resistant poliovirus permits the simultaneous multiplication of guanidine-dependent mutants of poliovirus in rescue experiments in the absence of the drug (Ikegami *et al.*, 1964). Conversely, guanidine-dependent or guanidine-resistant poliovirus can rescue guanidine-sensitive poliovirus in 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. The simplest hypothesis to explain this negative result is that the drug-requiring process of the virus strains used involves the same virus-directed protein or proteins.

Rescue experiments have also been carried out with HBB-dependent coxsackievirus A9 and drug-sensitive echovirus 7, and rescue of the dependent virus in the absence of HBB has been demonstrated. Not all

virus combinations have resulted in virus rescue. For example, no significant rescue of HBB-sensitive echovirus 7 by HBB-dependent coxsackievirus A9 could be demonstrated in HBB-treated cultures (Ikegami *et al.*, 1964). Similarly, guanidine-sensitive poliovirus 2 was not rescued by a guanidine-resistant poliovirus 1 in the presence of guanidine, although the same type 2 virus could be rescued by guanidine-dependent virus. The reasons for this are not clear.

To explain rescue, it has been postulated that the virus RNA polymerase or some other essential component synthesized under the direction of one virus can function in the replication of the RNA of a heterologous virus, such as of a drug-requiring virus in the absence of the drug, or drug-inhibited virus in the presence of drug. Doubly infected cultures yield twenty-five times greater amounts of assisting than rescued virus. The predominance of assisting over rescued virus particles is also reflected in the finding that the bulk of the capsid proteins of the rescued virus is provided by the assisting virus (Ikegami *et al.*, 1964; Holland and Cords, 1964). The low yield of rescued virus RNA and virus does not necessarily mean that the homologous enzyme or other essential component is less efficient in the synthesis of heterologous enterovirus RNA. This phenomenon could be explained on the assumption that most of the polymerase or some other component is occupied by homologous RNA.

SYNERGIC EFFECTS AND BLOCKING OF ANTIVIRAL ACTION

Combined treatment with HBB and guanidine has proven much more effective than treatment with either compound alone in suppressing enteroviruses in multiple-cycle experiments (Tamm and Eggers, 1962). Figure 26 illustrates the marked and prolonged inhibition of echovirus 5 cytopathic effects by HBB and guanidine, given in combination. At low concentration neither compound has a significant effect alone, but there is marked and prolonged inhibition by a combination of 40 μM HBB and 300 μM guanidine. There is evidence that the basis for increased effectiveness of HBB and guanidine given in combination is two-fold, and involves synergic action as well as reduced probability of emergence of resistant mutants.

Synergic action of HBB and guanidine has been investigated in single-cycle growth experiments with coxsackievirus A9 in monkey kidney cells (Eggers and Tamm, 1963b). Table 12 shows that HBB (19 μM) or guanidine (219 μM) has no effect on virus multiplication when given alone. At 2.25-fold higher concentration, either compound markedly reduces virus

yield, but still higher concentrations are required to suppress virus multiplication completely. On the other hand, 19 μM HBB and 98 μM guanidine given together inhibit virus growth completely. The complete inhibition obtained by combined treatment cannot be explained on the basis of inhibition of mutants resistant to one compound but sensitive to the other, as neither compound is by itself inhibitory at the concentration used. The synergic inhibitory effect of HBB and guanidine on virus multiplication suggests that the primary sites of action of these inhibitors are not identical. This view is supported also by the fact that the virus-inhibitory spectra of HBB and guanidine are significantly different (Tamm and Eggers, 1962).

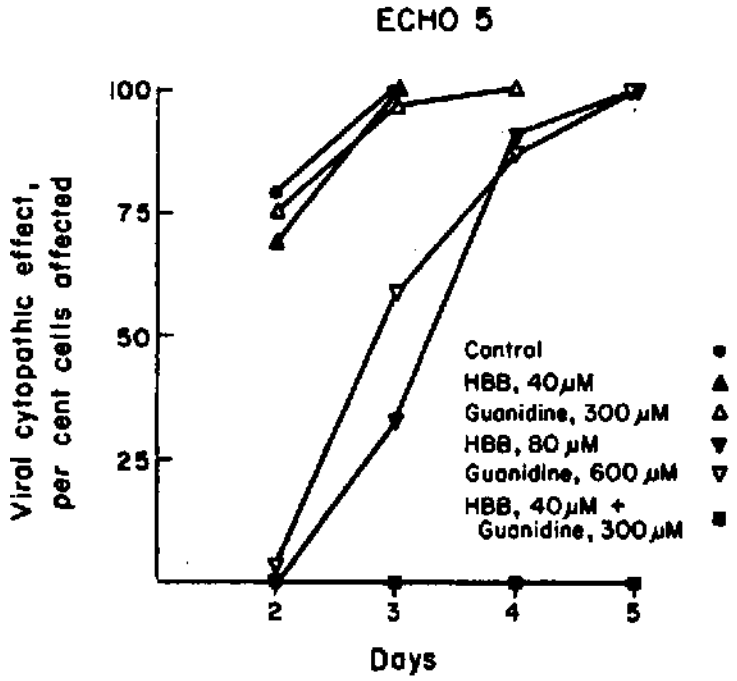


FIG. 26. Inhibition of echovirus 5 cytopathic effects by combined treatment with HBB and guanidine·HCl. Virus inoculum: 1600 $TCID_{50}$ per culture. (From Tamm and Eggers, 1962.)

However, it should be emphasized that HBB and guanidine inhibit the same virus-specific process, i.e. the synthesis of virus RNA.

To determine cross resistance between HBB and guanidine, variants of poliovirus 2, resistant to either HBB or guanidine, have been prepared by growing the virus in the presence of one or the other compound (Tamm and Eggers, 1962). The resistant variants were plaque-purified in the presence of HBB or guanidine. Experiments have been carried out with the

two compounds and clonal populations of resistant variants to determine whether resistance to one compound is associated with resistance to the other. It was found that the HBB-resistant variant prepared by passing the parent strain in the presence of HBB was 4.7 times less sensitive to HBB than the parent; on the other hand, it was only 1.5 times less sensitive to guanidine. The guanidine-resistant variant was 2.6 times less sensitive to guanidine than the parent population, but it was only 1.3 times less sensitive to HBB than the parent population. These results indicate that there is partial reciprocal cross-resistance between HBB and guanidine.

TABLE 12. SYNERGIC EFFECT OF HBB AND GUANIDINE ON THE MULTIPLICATION OF COXSACKIEVIRUS A9

Compound	Concentration, μM	Plaque-forming units/ml	Treated, % of untreated control
None	—	3.0×10^8	100
HBB	19	3.0×10^8	100
	43	6.5×10^6	2.2
	98	1.6×10^5	0.05
Guanidine	219	3.5×10^8	117
	493	1.1×10^6	0.4
	1110	1.4×10^5	0.05
HBB + Guanidine	19		
	98	2.0×10^5	0.07

From Eggers and Tamm, 1963b.

On the basis of all the evidence that HBB inhibits picornavirus multiplication through a virus-specific mechanism, it is not likely that the action of HBB on virus RNA synthesis could be blocked by metabolic precursors of RNA (Eggers and Tamm, 1962a). It is therefore not surprising that uridine, cytidine, adenosine and guanosine have failed to block the action of HBB, as HBB is probably not an antagonist of a metabolite required for the synthesis of both host cell and virus RNA (Eggers and Tamm, 1962a).

The fact that certain amino-acids and choline block the virus-inhibitory action of guanidine is of great interest, but unknown significance (Dinter and Bengtsson, 1964). In parallel experiments in which the action of guanidine on the multiplication of a bovine enterovirus could be blocked by lactalbumin hydrolysate, the virus-inhibitory action of HBB could not be blocked (Dinter and Bengtsson, 1964).

PROTECTIVE EFFECTS IN ANIMALS

HBB has not shown marked protective effects in mice or monkeys experimentally infected with poliovirus (Hollinshead and Smith, 1958; Fara and Cochran, 1963). Virus isolated from the brain or spinal cord of treated monkeys is resistant to HBB (Fara and Cochran, 1963). Similar results have been reported for guanidine (Barrera-Oro and Melnick, 1961). No experiments have been reported on combined effects of HBB and guanidine in animals, and the compounds have not been used in human medicine.

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CHAPTER 3

GUANIDINE

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INTRODUCTION AND HISTORY

GUANIDINE selectively inhibits the multiplication of small, lipid-free, RNA-containing animal viruses, the picornaviruses. The action of guanidine is in many ways similar to that of 2-(α-hydroxybenzyl)benzimidazole (HBB), a selective virus inhibitor discussed in the preceding chapter.

Guanidine hydrochloride is a resonance hybrid with a net positive charge (Fig. 1). At concentrations of 5 M or higher, this chemical is a

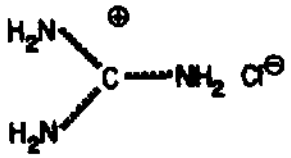

FIG. 1. Guanidine·HCl.

protein denaturant, but at low concentrations, such as 0.1–1.5 mM, it selectively inhibits the synthesis of the virus RNA of sensitive picornaviruses. Inhibition of production of infective virus appears to be secondary to inhibition of synthesis of virus RNA. The relationship between concentration of guanidine·HCl and reduction in yield of poliovirus types 1 and 2 is illustrated in Fig. 2. At a concentration of 0.4 mM, virus yield is reduced by more than 99%, and at higher concentrations of the drug the reduction is even greater.

There is considerable evidence that neither cell metabolism (Holland, 1963; Brown *et al.*, 1966) nor cell morphology (Rightsel *et al.*, 1961; Crowther and Melnick, 1961; Ueda *et al.*, 1961; Loddo *et al.*, 1962a) are significantly altered by guanidine at the low concentrations sufficient to inhibit the multiplication of sensitive viruses. Furthermore, guanidine does not inactivate virus particles when added to virus suspensions (Crowther and Melnick, 1961; Ueda *et al.*, 1961; Loddo *et al.*, 1962a).

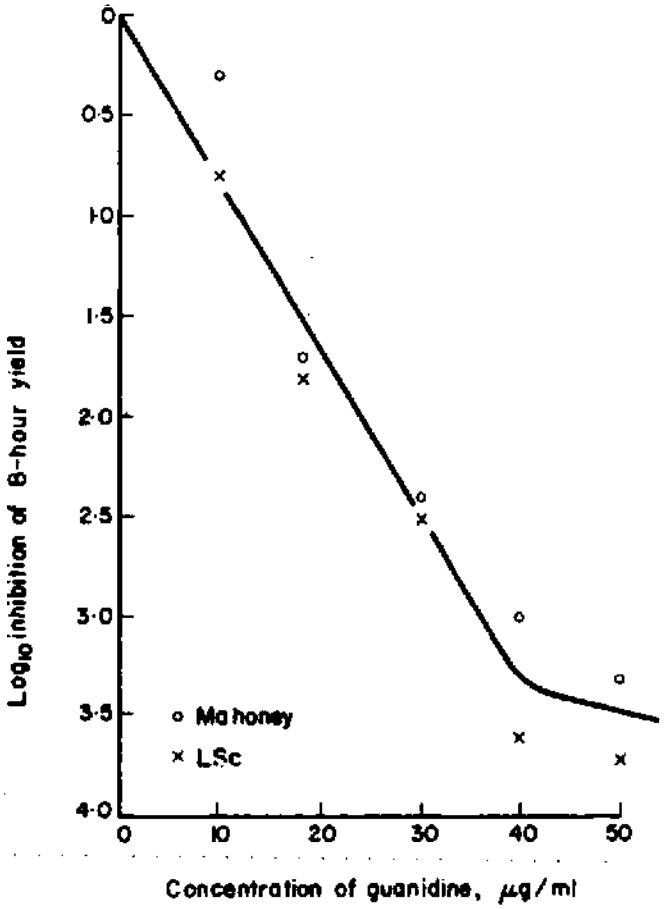

FIG. 2. Relationship between guanidine concentration and single-cycle yield of poliovirus from primary monkey kidney cultures. (From Crowther and Melnick, 1961.)

The antiviral effect of guanidine was detected through studies of more complex compounds which possessed virus-inhibitory activity, as well as through examination of guanidine itself. Rightsel and co-workers found in 1961 that the guanidine moiety was responsible for poliovirus-inhibitory activity of the guanidine salt of hydroxyaminomethylene malononitrile. Loddo and co-workers (1962a) examined guanidine, a series of derivatives

and other compounds and found that guanidine hydrochloride was the most active in inhibiting poliovirus multiplication. Results which suggested that the antiviral effects of 2-imino-5-methylhexahydro-1,3,5-triazine were due to a decomposition product led Ueda and co-workers (1961) to demonstrate the inhibitory activity of guanidine on the multiplication of poliovirus.

The selective inhibition of picornavirus multiplication by guanidine provides an effective approach to the investigation of virus-specific processes in the infected cells. Use of selective virus inhibitors such as guanidine and HBB have advanced the understanding of virus-induced inhibition of cell metabolism, translation and transcription of virus RNA, and assembly of virus particles. The virus selectivity of guanidine and HBB has also been useful in virus identification and subgrouping of picornaviruses (Tamm and Eggers, 1962). Guanidine is not useful in chemotherapy of virus infections in animals; however, elucidation of the precise molecular site of action of guanidine may suggest an effective approach to antiviral chemotherapy.

VIRUS-INHIBITORY SPECTRUM

Guanidine selectively inhibits the multiplication of many but not all of the picornaviruses (Table 1). Among the picornaviruses, the human enteroviruses represent the bulk of guanidine-sensitive viruses. All three types of poliovirus are guanidine-sensitive and there is no significant difference between the virulent and attenuated strains in their sensitivity to guanidine. All coxsackievirus types examined have proven susceptible to inhibition by guanidine. Several echovirus types also are sensitive. There is wide variation in the susceptibility of foot-and-mouth disease virus to inhibition by guanidine. When tested in Earle's salt solution in Waymouth medium, Pringle (1964) found types SAT1, SAT2, SAT3, and Asia 1 to be most susceptible, whereas types A, C, and O were less sensitive. Dinter and Bengtsson (1964) found that only the attenuated strains of types A_4 and O_3 of foot-and-mouth disease virus were markedly inhibited by guanidine in Eagle's minimal essential medium, whereas both virulent and attenuated strains were susceptible to inhibition by guanidine when tested in Earle's salt solution.

Only a few rhinoviruses have so far been examined for sensitivity to guanidine, although extensive studies have been carried out with HBB (cf. preceding chapter). Rhinovirus type 2 (strain HGP) is sensitive to guanidine (Table 1), whereas type 1B (strain B632) is insensitive (Table 2).

TABLE 1. VIRUSES INHIBITED BY GUANIDINE[a]

Virus	Type	Strain	Cell culture	Medium[b]	Guanidine mM	Reference
Polio	1	Brunhilde	Monkey kidney	LH	1.0	Loddo *et al.* (1962a)
Polio	1	Mahoney	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Polio	1	Mahoney	Monkey kidney	LH	0.5	Crowther and Melnick (1961)
Polio	1	Mahoney	HeLa	YLE	1.0	Ueda *et al.* (1962)
Polio	1	LSc	Monkey kidney	MEM	0.2	Tamm and Eggers (1962)
Polio	1	LSc	Monkey kidney	LH	0.5	Crowther and Melnick (1961)
Polio	2	MEF_1	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Polio	2	MEF_1	Monkey kidney	MEM	0.21	Tamm and Eggers (1962)
Polio	2	MEF_1	Monkey kidney	LH	1.0	Loddo *et al.* (1962a)
Polio	2	MEF_1	HeLa	YLE	1.0	Ueda *et al.* (1962)
Polio	2	P712	Monkey kidney	LH	0.5	Crowther and Melnick (1961)
Polio	3	Saukett	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Polio	3	Saukett	HeLa	YLE	1.0	Uedo *et al.* (1962)
Polio	3	Saukett	Monkey kidney	LH	1.0	Loddo *et al.* (1962a)
Polio	3	Leon	Monkey kidney	LH	0.5	Crowther and Melnick (1961)
Echo	1	Farouk	Monkey kidney	MEM	0.46	Tamm and Eggers (1962)
Echo	5	Noyce	Monkey kidney	MEM	0.68	Tamm and Eggers (1962)
Echo	6	D'Amori	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Echo	9	Bourn	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Echo	12	Travis	Monkey kidney	MEM	1.5	Halperen *et al.* (1964)
Coxsackie	A7	AB IV Habel	Monkey kidney	MEM	0.47	Tamm and Eggers (1962)
Coxsackie	A9	Grigg	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Coxsackie	A9	Woods	Monkey kidney	MEM	1.1	Eggers and Tamm (1963)
Coxsackie	A11	D 52148	HEL	RMEM	0.67	Tamm and Eggers (1962)
Coxsackie	A13	D 5359	HEL	RMEM	0.27	Tamm and Eggers (1962)
Coxsackie	A16	Toronto	Monkey kidney	MEM	0.21	Tamm and Eggers (1962)
Coxsackie	A18	D 52112	HEL	RMEM	0.12	Tamm and Eggers (1962)

TABLE 1 (*cont.*)

Virus	Type	Strain	Cell culture	Medium[b]	Guanidine mM	Reference
Coxsackie	B1	Conn-5	H.Ep. 2	ESS	1.25	Rightsel *et al.* (1961)
Coxsackie	B3	Nancy	Monkey kidney	MEM	0.73	Tamm and Eggers (1962)
Coxsackie	B5	Faulkner	Monkey kidney	MEM	0.64	Tamm and Eggers (1962)
Foot-and-mouth disease	A	119-1	Pig kidney	EW	2.0–6.0	Pringle (1964)
Foot-and-mouth disease	A_4	virulent	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Foot-and-mouth disease	A_4	attenuated	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Foot-and-mouth disease	C	C 997-1	Pig kidney	EW	2.0–6.0	Pringle (1964)
Foot-and-mouth disease	C	virulent	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Foot-and-mouth disease	C	attenuated	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Foot-and-mouth disease	O	M11-30	Pig kidney	EW	2.0–6.0	Pringle (1964)
Foot-and-mouth disease	O_3	virulent	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Foot-and-mouth disease	O_3	attenuated	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Foot-and-mouth disease	SAT 1	TuR 43/62-1	Pig kidney	EW	1.0	Pringle (1964)
Foot-and-mouth disease	SAT 2	Rho 1-285/1	Pig kidney	EW	1.0	Pringle (1964)
Foot-and-mouth disease	SAT 2	Kenya 3-476	Pig kidney	EW	1.0	Pringle (1964)
Foot-and-mouth disease	SAT 3	RV 7-323	Pig kidney	EW	1.0	Pringle (1964)
Foot-and-mouth disease	Asia 1	Pak 1-1	Pig kidney	EW	1.0	Pringle (1964)
Bovine enterovirus		G-UP 6976	Calf kidney	ESS	1.0	Dinter and Bengtsson (1964)
Rhino		HGP	HEL	RMEM	0.56	Tamm and Eggers (1962)
Measles		Edmonston	FL	YLE	1.0	Ueda *et al.* (1962)
Measles		Edmonston	HeLa	YLE	1.0	Ueda *et al.* (1962)

[a] The criterion for inhibition of virus growth was 75% or greater inhibition of virus-induced cytopathic effects or virus yield.

[b] Medium: LH—lactalbumin in Hanks' salt solution; ESS—Earle's salt solution + monkey serum; YLE—yeast extract and lactalbumin in Earle's salt solution + bovine serum; MEM—Eagle's minimum essential medium; RMEM—reinforced Eagle's minimum essential medium; EW—Earle's salt solution + Waymouth medium + ox serum.

TABLE 2. VIRUSES NOT INHIBITED BY GUANIDINE

Virus	Cell culture	Medium[a]	Reference
Picornavirus			
Echo 22	Monkey kidney	MEM	Tamm and Eggers (1962)
Echo 23	Monkey kidney	MEM	Tamm and Eggers (1962)
Rhinovirus B632	HEL	RMEM	Tamm and Eggers (1962)
GDVII	BHK21	RMEM	Sturman and Tamm (1962)
Reovirus	H.Ep. 2	ESS	Rightsel *et al.* (1961)
Arbovirus A			
Eastern equine encephalitis	H.Ep. 2	ESS	Rightsel *et al.* (1961)
Arbovirus B			
St. Louis encephalitis	Detroit 6	ESS	Rightsel *et al.* (1961)
Japanese B encephalitis	HeLa	YLE	Ueda *et al.* (1962)
Myxovirus			
Influenza A2	Monkey kidney	ESS	Rightsel *et al.* (1961)
Parainfluenza 1 (Sendai)	Monkey kidney	ESS	Rightsel *et al.* (1961)
Parainfluenza 3 (HA 1)	Monkey kidney	ESS	Rightsel *et al.* (1961)
Simian parainfluenza (SV5)	Monkey kidney	MEM	Choppin and Holmes (1967)
Measles (Edmonston)	H.Ep. 2	ESS	Rightsel *et al.* (1961)
Measles (Edmonston)	HeLa	?	Rustigian (personal communication, 1964)
Papovavirus			
Polyoma	C3H	ESS	Rightsel *et al.* (1961)
Adenovirus			
Types 1, 2, 3, 4, 5, 6, 7	FL	YLE	Ueda *et al.* (1962)
Herpesvirus			
Herpes simplex	H.Ep. 2, monkey kidney	ESS	Rightsel *et al.* (1961)
Herpes B	H.Ep. 2	ESS	Rightsel *et al.* (1961)
Poxvirus			
Vaccinia	H.Ep. 2	ESS	Rightsel *et al.* (1961)

[a] Medium: ESS—Earle's salt solution + monkey serum; YLE—yeast extract and lactalbumin in Earle's salt solution + bovine serum; MEM—Eagle's minimum essential medium; RMEM—reinforced Eagle's minimum essential medium.

Several other picornaviruses are not susceptible to guanidine (Table 2). Echovirus types 22 and 23 differ from the other echoviruses in that they are not inhibited by either guanidine (Tamm and Eggers, 1962) or HBB (Eggers and Tamm, 1961), and these viruses produce morphological changes in the cells which also differ from those of other echoviruses (Shaver *et al.*, 1958). The murine picornaviruses represented by GD VII (Table 2) are also resistant to inhibition by guanidine (Sturman and Tamm, 1966).

In general, members of the other major groups of viruses are not inhibited by guanidine at concentrations that do not significantly alter cell metabolism (Table 2). RNA-containing viruses which are not susceptible to guanidine at low concentrations include arboviruses, myxoviruses and reoviruses (Rightsel *et al.*, 1961; Ueda *et al.*, 1962). The list of DNA-containing viruses not inhibited selectively by guanidine includes herpesviruses, papovaviruses (Rightsel *et al.*, 1961), adenoviruses (Ueda *et al.*, 1962) and poxviruses (Rightsel *et al.*, 1961).

There are some reports of viruses other than picornaviruses which are sensitive to guanidine. The multiplication of the Edmonston strain of measles virus has been reported to be inhibited (Ueda *et al.*, 1962); however this has not been confirmed by other laboratories in which the same strain of measles virus was examined (Rightsel *et al.*, 1961; Rustigian,

TABLE 3. INHIBITION OF ENTEROVIRUS MULTIPLICATION BY GUANIDINE

Virus	Type	Strain	Virus yield[a]		% inhibition
			No guanidine	0.15 mM guanidine	
Polio	2	MEF_1	$10^{7.5}$	$10^{6.5}$	90%
Polio	3	Saukett	$10^{7.5}$	$10^{6.9}$	75%
Polio	1	Mahoney	$10^{8.5}$	$10^{8.0}$	68%
Coxsackie	B1	Connecticut 5	$10^{6.8}$	$10^{6.3}$	68%
Echo	6	D'Amori	$10^{7.3}$	$10^{7.0}$	50%
Coxsackie	A9	Geigy	$10^{8.7}$	$10^{8.5}$	39%
Echo	9	Bourn	$10^{8.0}$	$10^{7.8}$[b]	39%

[a] Guanidine added at time of inoculation of H.Ep. 2 cells in Earle's salt solution + monkey serum. Virus yield expressed as $TCID_{50}$/ml after 7 days at 37°C.

[b] Concentration of guanidine was 0.30 mM.

From Dixon, Rightsel and Skipper, 1965.

personal communication, 1964). There is also one report that the multiplication of an oncogenic virus, avian myeloblastosis virus, is sensitive to inhibition by guanidine (Lacour *et al.*, 1965), but so far this has not been confirmed. An RNA-containing plant virus, tobacco necrosis virus, has been reported to be inhibited by 0.015 M guanidine carbonate applied to infected plant leaves without detectable toxicity to the plant (Varma, 1968). It is difficult to evaluate the significance of these observations.

The effectiveness of guanidine in reducing the yield of picornaviruses is illustrated by results summarized in Table 3, in which viruses are arranged in a descending order of sensitivity to 0.15 mM guanidine · HCl. Among the seven enteroviruses examined, the most sensitive virus, polio 2 (MEF1 strain) is inhibited by 90%, whereas at the other end of the spectrum, echo 9 (Bourn strain) is inhibited by 39%. It is well known that polioviruses are among the most sensitive picornaviruses with respect to inhibition by guanidine (Rightsel *et al.*, 1961; Loddo *et al.*, 1962a; Tamm and Eggers, 1962). At concentrations of guanidine higher than 0.15 mM, most of the enteroviruses are inhibited by more than 90%, which makes quantitative comparisons unsatisfactory.

In Table 4 the sensitivity of picornaviruses to guanidine is compared in terms of the concentration of compound required to reduce the extent of

TABLE 4. INHIBITION OF VIRUS CYTOPATHIC EFFECTS BY GUANIDINE

Virus	Type	Strain	75% protective concentration, mM[a]
Coxsackie	A18	D5112	0.12
Polio	1	LSc	0.20
Polio	2	MEF_1	0.21
Coxsackie	A16	Toronto	0.21
Coxsackie	A13	D5359	0.27
Echo	1	Farouk	0.46
Coxsackie	A7	AB IV Habel	0.47
Coxsackie	B5	Faulkner	0.52
Rhino		HGP	0.56
Coxsackie	A11	D52148	0.67
Echo	5	Noyce	0.68
Coxsackie	B3	Nancy	0.73

[a] Based on observations made on the third day after virus inoculation of primary cultures of rhesus monkey kidney cells in Eagle's minimum essential medium.

From Tamm and Eggers, 1962.

viral cytopathic effects by 75%. The data are from a study by Tamm and Eggers (1962) in which the sensitivity of many picornaviruses to guanidine and HBB was compared. It can be seen again that polioviruses are among the most sensitive viruses with respect to inhibition by guanidine. In contrast (cf. Table 5), polioviruses are less sensitive to HBB than echoviruses or group B coxsackieviruses. It is also known that most group A coxsackieviruses, while resistant to HBB, are still sensitive to guanidine.

TABLE 5. INHIBITION OF VIRUS CYTOPATHIC EFFECTS BY 2-(α-HYDROXYBENZYL)BENZIMIDAZOLE (HBB)

Virus	Type	Strain	75% protective concentration, mM[a]
Coxsackie	B5	Faulkner	0.064
Echo	5	Noyce	0.068
Echo	1	Farouk	0.070
Coxsackie	B3	Nancy	0.077
Polio	2	MEF_1	0.200
Polio	1	LSc	0.440

[a] Based on observations made on the third day after virus inoculation of primary cultures of monkey kidney cells in Eagle's minimum essential medium.

From Tamm and Eggers, 1962.

Although there are such distinct differences in the virus-inhibitory spectra of HBB and guanidine, the biochemical mechanism of action of these selective virus inhibitors appears to be closely similar (cf. later sections of this chapter). The differences in spectra probably reflect genetically determined differences in the fine structure of the virus-specific target molecules.

The concentration at which guanidine causes 75% inhibition of cytopathic effects of the MEF1 strain of poliovirus type 2 is 0.21 mM (Table 4), whereas that required to reduce the yield of virus by 90% is only 0.15 mM (Table 3). Similar observations have also been made with a number of benzimidazole derivatives (cf. preceding chapter). Extensive studies by Bablanian and co-workers (Bablanian *et al.*, 1965a, b) provide evidence to support the view that inhibition of viral cytopathic effects by guanidine is due to inhibition of virus multiplication (*vide infra*).

The medium in which compounds are tested is an important variable, which must be considered in evaluating the antiviral activity of guanidine.

The antiviral activity of guanidine is reduced when infected cells are incubated in Eagle's minimal medium as compared to media containing amino-acids and other components at lower concentrations (Dinter and Bengtsson, 1964; Lwoff and Lwoff, 1964b). The data summarized in Table 6 illustrate the effects of medium on the inhibition of foot-and-mouth disease virus multiplication by guanidine. In these experiments virus yields from calf kidney cells in either Earle's salt solution with 2% calf serum or Eagle's minimum essential medium (Eagle, 1959) were determined. Eagle's minimum essential medium suppresses guanidine inhibition as compared to the inhibition obtained in Earle's salt solution; however, the degree of suppression varies greatly among strains. For example, Eagle's medium suppresses the effects of guanidine on the multiplication of virulent A_4 strain to a much greater degree than the attenuated A_4 strain.

TABLE 6. EFFECT OF MEDIUM ON INHIBITION OF VIRUS YIELD BY GUANIDINE

	Yield of virus in calf kidney cells[a]			
	Earle's salt solution + 2% calf serum		Eagle's minimum essential medium	
Virus strain	No guanidine	1.0 mM guanidine	No guanidine	1.0 mM guanidine
FMDV A_4 virulent	$10^{6.0}$	$10^{2.7}$	$10^{6.8}$	$10^{5.4}$
FMDV A_4 attenuated	$10^{6.8}$	$< 10^{1.5}$	$10^{6.8}$	$10^{3.0}$
FMDV C virulent	$10^{5.5}$	$< 10^{1.5}$	$10^{6.0}$	$10^{3.5}$
FMDV C attenuated	$10^{6.6}$	$< 10^{1.5}$	$10^{6.8}$	$10^{5.0}$
FMDV O_3 virulent	$10^{5.0}$	$< 10^{1.5}$	$10^{6.0}$	$10^{4.8}$
FMDV O_3 attenuated	$10^{5.5}$	$< 10^{1.5}$	$10^{6.1}$	$10^{1.8}$
G-up	$10^{5.5}$	$10^{3.75}$	$10^{6.0}$	$10^{2.5}$

[a] $TCID_{50}$/0.1 ml 30 hr after infection.
From Dinter and Bengtsson, 1964.

Eagle's medium consists of 13 amino-acids and 8 vitamins in Earle's salt solution (Eagle, 1959). By investigating the effects of each individual amino-acid and vitamin on the inhibitory action of guanidine it has been found that only four amino-acids, methionine, valine, leucine, and threonine, are effective in antagonizing guanidine inhibition (Dinter and

Bengtsson, 1964). Any medium which contains these amino-acids may block inhibition by guanidine. The blocking effect depends on the concentrations of these amino-acids. This interesting phenomenon is discussed in greater detail in a later section.

STRUCTURE–ACTIVITY RELATIONSHIPS

Structural requirements for the antiviral activity of guanidine are stringent: structural modification of the molecule either reduces or eliminates activity. Substituted guanidines such as methylguanidine and aminoguanidine (Loddo *et al.*, 1962a; Lwoff and Lwoff, 1964a) are less effective than guanidine salts, but nevertheless do reduce virus yield. In general, most of the simple salts of guanidine such as guanidine hydrochloride (Rightsel *et al.*, 1961), guanidine nitrate (Ueda *et al.*, 1961), and guanidine hydrosulfate (Ferrari *et al.*, 1965) have similar selective inhibitory activity. This is illustrated in Table 7. Related compounds such as carbonylglycine and creatine do not possess selective antiviral activity (Loddo *et al.*, 1962a; Lwoff and Lwoff, 1964a). Urea is another related compound which has been investigated; however, there are conflicting reports as to its selective inhibitory action on poliovirus multiplication. Urea is much more cytotoxic than guanidine (Loddo *et al.*, 1962a), but it can be used at 0.1–0.3 M concentrations for short periods without killing normal KB cells (Lwoff, 1962). At these concentrations of urea, Lwoff and co-workers (1963b) found inhibition of poliovirus multiplication, but urea could not replace guanidine in the multiplication of guanidine-dependent mutants. However, Crowther and Melnick (1961) reported that urea had no inhibitory effect on poliovirus multiplication. It appears that if urea does inhibit poliovirus multiplication, the mechanism of action of urea is unrelated to that of guanidine and is unselective.

More complex guanidine derivatives have been studied extensively in an effort to find a compound effective in chemotherapy of virus disease and to elucidate the mechanism of action of guanidine (Loddo *et al.*, 1964b; Loddo and Gessa, 1965; Sato *et al.*, 1966; Ueda *et al.*, 1966). Results of a study by Ferrari and co-workers (1965) are presented in Table 7. The effectiveness of various compounds is related to guanidine hydrochloride as the reference compound. Relative selectivity refers to the ratio of relative inhibitory activity to relative toxicity (Tamm, 1956; Tamm *et al.*, 1961). Methylglyoxal*bis*-guanylhydrazone (methyl-GAG) is the only complex guanidine derivative which, while more active, possesses the same selectivity ratio as the simple guanidine salts (Table 7). The antiviral

TABLE 7. INHIBITORY ACTIVITY AND TOXICITY OF GUANIDINE DERIVATIVES

Compound	Molecular weight	Minimal inhibitory concentration[a] (mM)	Relative inhibitory activity	Cytotoxic concentration[b] (mM)	Relative toxicity	Relative selectivity
Guanidine·HCl	97	0.10	1.0	10.3	1.0	1
Guanidine·H_2SO_4	157	0.06	1.7	6.35	1.63	1.05
Guanidine·H_2PO_4	157	0.32	0.31	3.18	3.24	0.096
Methylglyoxal*bis*-guanylhydrazone	186	0.017	5.8	0.043	5.8	1
Pyrimidine-4-guanidine 1/2 H_2SO_4	188	1.33	0.075	10.65	0.98	0.075
2-6-dimethylpyrimidine-4-guanidine 1/2 H_2SO_4	218	0.76	0.13	4.59	2.24	0.058
O-chlorobenzyl-N_1, N_2-dimethylguanidine	260	0.19	0.53	0.77	13.4	0.0395
N-benzyl-N_1, N_2-dimethylguanidine	226	0.22	0.45	0.89	11.6	0.039
Sulphonniazide	309	6.45	0.016	16.2	0.64	0.025

[a] Concentration which produced at least 50% inhibition reduction of poliovirus yield 36 hr after infection measured as $TCID_{50}$/ml.
[b] Human amnion cells (Mascoli line) incubated for 5 days at 37°C.
From Ferrari, Loddo and Gessa, 1965.

activity of this compound differs from that of guanidine in several ways: (1) it inhibits vaccinia virus multiplication, which is resistant to guanidine; (2) guanidine-resistant poliovirus is quite susceptible to inhibition by methyl-GAG; (3) methyl-GAG does not support the multiplication of guanidine-dependent poliovirus (Ferrari *et al.*, 1964). The activity of the other complex derivatives is limited and the virus inhibitory spectrum of these compounds is similar to that of guanidine. The effectiveness of the other complex derivatives is probably due to the presence of a guanidine moiety or formation of guanidine upon decomposition.

EFFECTS ON NORMAL CELLS

Guanidine does not alter the structure or functions of normal cells when used at concentrations sufficient to inhibit the replication of sensitive viruses. This in itself is strong evidence that the action of guanidine on virus growth is selective and unlike the action of metabolic inhibitors, such as amino-acid or base analogs, which inhibit virus multiplication unselectively. Concentrations of guanidine hydrochloride as high as 2 mM produce only slight cytotoxic changes in monkey kidney cells after the seventh day of treatment (Crowther and Melnick, 1961). We have determined the effects of 1.1 mM guanidine hydrochloride on the division of S3 HeLa cells in suspension (Fig. 3). This high concentration of guanidine rapidly causes maximal inhibition of poliovirus development. However, the number of cells in uninfected cultures continues to increase exponentially even during treatment with guanidine. The total cell count after 70.5 hr in the presence of 1.1 mM guanidine is only 20% lower than that in control untreated cultures. The effect of 0.22 mM 2-(α-hydroxybenzyl)-benzimidazole, another selective virus inhibitor, is similarly slight. Thus, neither guanidine nor HBB significantly affects the exponential rate of division of uninfected cells under conditions under which multiplication of sensitive viruses is markedly inhibited.

Investigations of the effects of guanidine on cell metabolism have been limited, but the results have been consistent in indicating that guanidine does not have significant effects. Guanidine at a concentration of 1.5 mM does not affect the normal pH changes which take place in uninfected monkey kidney cell cultures (Crowther and Melnick, 1961). Brown and co-workers (1966) have determined that the rate of incorporation of ^{14}C-valine into protein in normal baby hamster kidney cells in culture is not changed after incubation for 3 hr in the presence of 1–4 mM guanidine. Others have reported that concentrations as high as 8 mM

guanidine reduce incorporation of ^{14}C-glycine in chick embryo cells by 40% (Shirman *et al.*, 1967); however, this concentration is many times higher than necessary for selective virus inhibition. We have found that 1.5 mM guanidine does not alter the rate of RNA synthesis in uninfected HeLa cells after incubation for 2 hr (unpublished data).

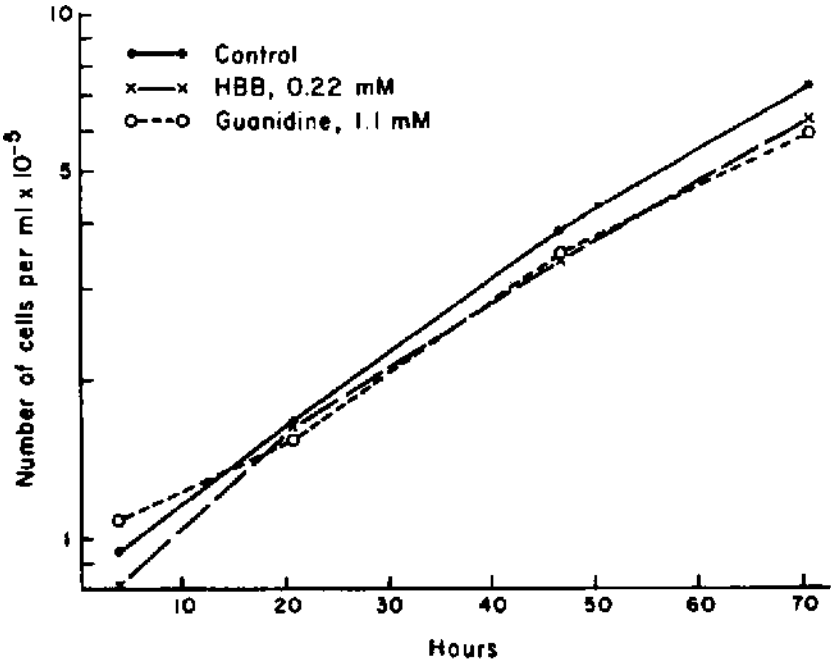

FIG. 3. Effects of guanidine and 2-(α-hydroxybenzyl)benzimidazole (HBB) on the multiplication of S3 HeLa cells in suspension.

A recent report by Graves (1969) suggests that guanidine at a concentration of 0.75 mM may inhibit incorporation of radioactive carbon from glucose into DNA and RNA in normal HeLa cells. There is considerable variation in the results of these experiments and no apparent correlation between concentration of guanidine and extent of inhibition. The weight of the evidence so far suggests that guanidine hydrochloride has no significant effect on normal cellular biosynthesis at virus-inhibitory concentrations.

KINETICS OF ANTIVIRAL ACTION

In the preceding section evidence was presented that guanidine inhibits virus multiplication at concentrations which have no effect on cellular

metabolism. There have been extensive studies to determine the virus-specific mechanism of action of guanidine. Guanidine does not inactivate the infectivity of poliovirus when the virus is treated with guanidine before inoculation (Crowther and Melnick, 1961; Loddo *et al.*, 1962a; Toyoshima *et al.*, 1963; Dixon *et al.*, 1965). In experiments of this kind poliovirus suspensions have been incubated at 37°C with 1 mM guanidine for periods up to 4 days without an effect on infectivity (Carp, 1964).

Most of the studies on the kinetics of action of guanidine during the virus growth cycle have been performed in poliovirus-infected HeLa cells. The normal growth cycle of poliovirus in these cells is shown in Fig. 4 (cf. reviews by Tamm and Eggers, 1965; Levintow, 1965). The initial step

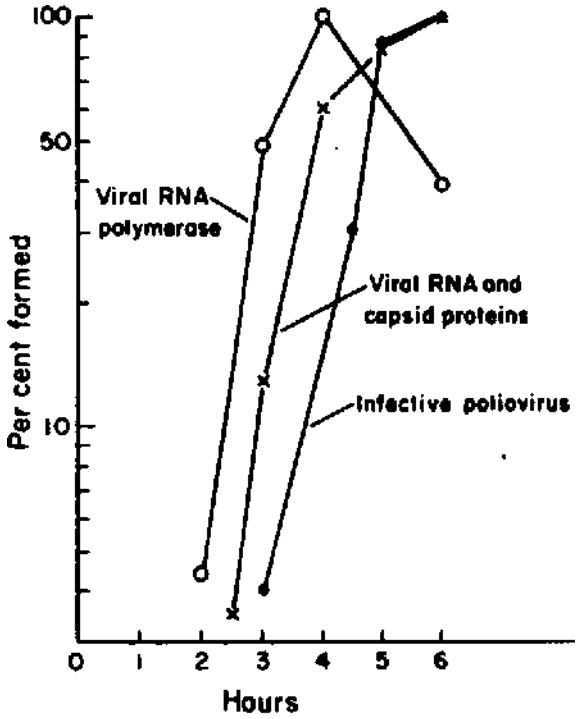

FIG. 4. Time course of poliovirus biosynthesis and formation of infective virus in HeLa cells. (From Tamm, 1968.)

in poliovirus infection is the adsorption of virus particles to specific receptor sites on the cell membrane. Following this, penetration occurs, and the genome of the virus, its single-stranded RNA, is released from the protein coat into the cytoplasm of the cell by a mechanism which is not fully understood. Mandel (1967) has investigated extensively the early interaction of poliovirus and HeLa cells. His results indicate that the virus particle may be engulfed by the cell at 21°C, whereas release of the

virus genome requires a higher temperature. Mandel (1967) suggests that the intact virion is taken into the cell within a plasma membrane-bounded vacuole, that uncoating begins inside the vacuole, and that finally the virus RNA is released into the cytoplasm of the cell.

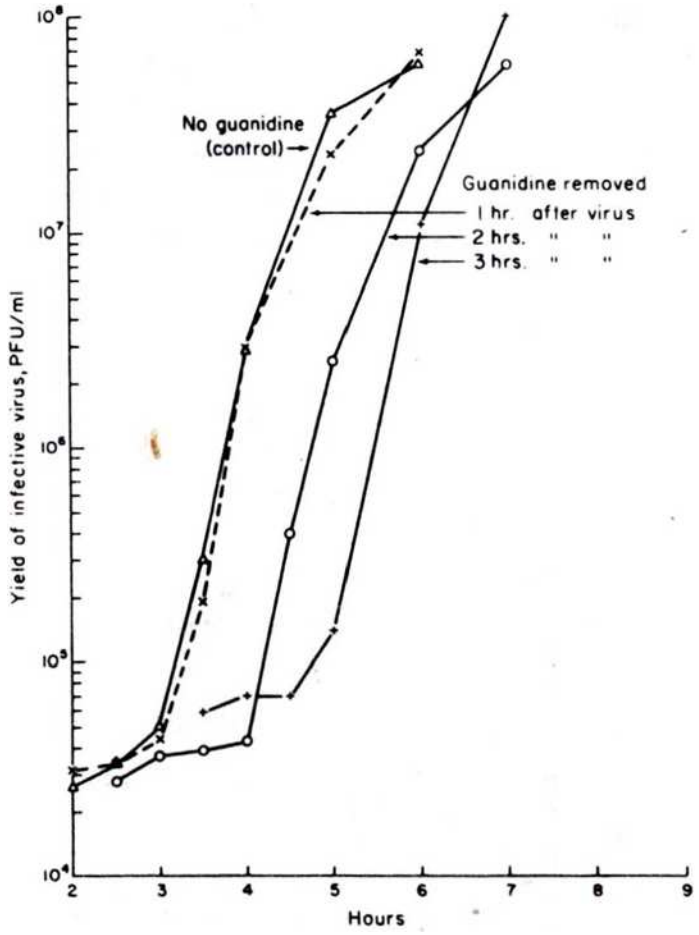

FIG. 5. Time course of the multiplication of guanidine-sensitive poliovirus type 1 in HeLa cells with 1 mM guanidine present for 1, 2, or 3 hr after inoculation. (From Eggers, Ikegami and Tamm, 1965a).

Once free in the cytoplasm, poliovirus RNA functions both as messenger in virus protein synthesis (Penman *et al.*, 1963; Summers and Levintow, 1965) and as template for a complementary strand of RNA, which in turn functions as template for progeny RNA (Baltimore, 1966; Baltimore and Girard, 1966; Bishop and Koch, 1967). The onset of virus RNA synthesis can, under special circumstances, be detected as early as 1 hr after infection (Baltimore *et al.*, 1966). The translation and transcription of virus RNA are temporally closely associated in the infected cell (Darnell *et al.*, 1961),

which is shown by the overlapping time courses of virus RNA and capsid protein synthesis (Fig. 4). Production of new virus particles becomes detectable shortly after the onset of virus biosynthesis, i.e. approximately 3 hr after infection (Baltimore *et al.*, 1966; Fig. 4). Within 2 hr after infection cell biosynthesis is significantly inhibited (Salzman *et al.*, 1959; Zimmerman *et al.*, 1963; Holland and Peterson, 1964; Bablanian *et al.*, 1965a); by this time both virus RNA synthesis (Baltimore *et al.*, 1966) and virus RNA polymerase activity (Baltimore *et al.*, 1963) are detectable.

Poliovirus RNA polymerase is thought to be a virus-specific enzyme, as it is found only in infected cells (Baltimore *et al.*, 1963). Although the enzyme has not been isolated or purified, enzymatic activity can be measured *in vitro* by the synthesis of RNA from nucleoside triphosphates utilizing cytoplasmic extracts from infected cells. The polymerase activity increases rapidly and reaches a peak at 4 hr and then declines (Fig. 4). Progeny virus RNA molecules are associated with virus capsid proteins to form mature virus particles and infectious virus is released from cells over a 20-hr period.

The early events of virus–cell interaction, such as adsorption and penetration, are not inhibited by guanidine (Crowther and Melnick, 1961; Toyoshima *et al.*, 1963; Carp, 1964; Eggers *et al.*, 1965a). Results obtained by Eggers and co-workers (Eggers *et al.*, 1963b; Eggers *et al.*, 1965a) have established that virus multiplication becomes sensitive to guanidine between 1 and 2 hr after virus inoculation. Figure 5 shows that there is no change in the virus growth cycle when guanidine is removed within 1 hr after HeLa cells are infected with drug-sensitive poliovirus. However, removal of guanidine at 2 hr or later is associated with a delay in the onset of multiplication of poliovirus. These data also illustrate the complete reversibility of the virus-inhibitory effect of guanidine in that the cultures from which guanidine has been removed produce as high a yield of poliovirus as the control untreated cultures. In analogous experiments with a mutant of poliovirus which requires guanidine for normal multiplication there is a normal time course of virus growth when guanidine is added 1 hr after inoculation (Fig. 6). However, addition of guanidine at 2 hr or later delays the onset of multiplication of this guanidine-dependent mutant of poliovirus. These results clearly establish that guanidine is required beginning 1 hr after inoculation for multiplication of guanidine-dependent poliovirus (Fig. 6; Eggers *et al.*, 1965a).

Further evidence to support the view that the process affected by guanidine begins 1 hr after inoculation is provided by results of UV irradiation experiments using the infective center technique (Eggers *et al.*,

1965b). The potential of infected cells to produce virus can be readily destroyed by UV irradiation if the cells are irradiated shortly after infection; however, the potentiality of cells to serve as infective centers becomes resistant to UV irradiation between 50 and 110 min after inoculation. This UV resistance does not develop if the infected cells are

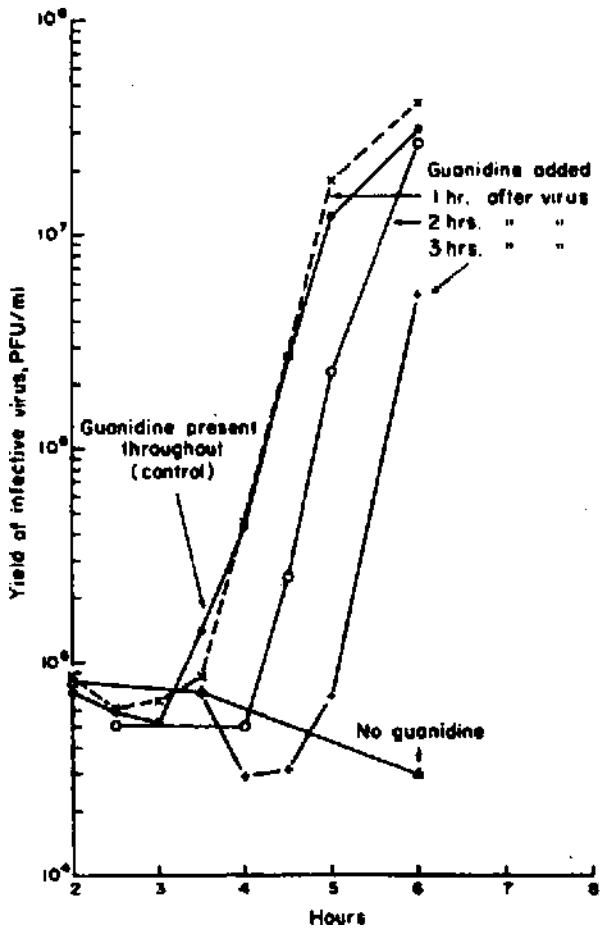

FIG. 6. Time course of the multiplication of guanidine-dependent poliovirus type 1 (Loddo) in HeLa cells. One mM guanidine was added at 1, 2, or 3 hr after inoculation. (From Eggers, Ikegami and Tamm, 1965a.)

treated with guanidine, and conversely guanidine is required for UV resistance to develop in cells inoculated with guanidine-dependent poliovirus. These results indicate that guanidine affects the process which is involved in the acquisition of UV resistance of infected cells between 50 and 110 min after infection. The onset of effectiveness of guanidine in these studies correlates well with the time of onset of action of guanidine in the virus growth cycle obtained from kinetic studies. Thus, the period

of sensitivity to guanidine begins between 1 and 2 hr after inoculation with guanidine-sensitive virus, and, conversely, the guanidine-requiring period in the multiplication of guanidine-dependent mutants also begins at this time.

The duration of the guanidine-sensitive period is a question which has produced some controversy. Eggers and co-workers (1963b) found that guanidine stops multiplication of guanidine-sensitive virus when added as late as 3¼ hr after infection, a time when 25% of the total virus had already been made. Furthermore, removal of guanidine 3¼ hr after infection with a guanidine-dependent mutant results in cessation of multiplication of this virus. These results obtained from mass culture experiments (Eggers *et al.*, 1963b) were later confirmed in single cell studies (Caliguiri *et al.*, 1965). Lwoff and Lwoff (1963) and Lwoff (1965), however, reported that the

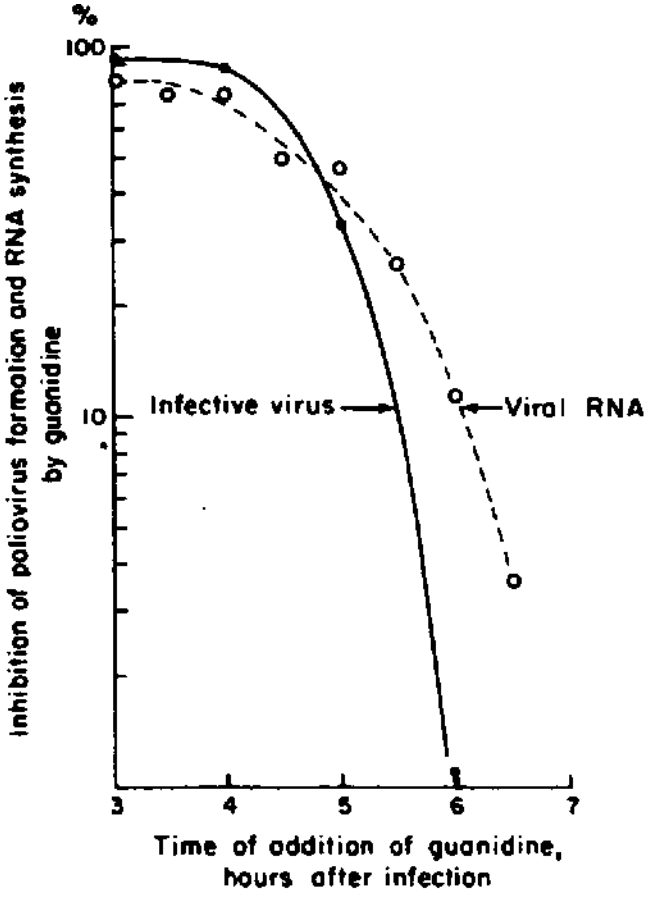

FIG. 7. Inhibition of poliovirus RNA synthesis and formation of infective virus by 1.5 mM guanidine added at various times after inoculation. (From Caliguiri and Tamm, 1968b.)

period during which guanidine is effective is very short (10 min) at the beginning of the exponential phase in virus growth. This was interpreted to mean that morphogenesis of virus RNA polymerase takes place within this short period and that guanidine interferes with this process (Lwoff, 1965). These results may have been affected by the presence of lactalbumin hydrolysate and other components in the rich medium. Lactalbumin hydrolysate has been shown to block the inhibitory effect of guanidine on virus multiplication (Dinter and Bengtsson, 1964; Lwoff and Lwoff, 1964b).

Figure 7 shows the results of a study in which guanidine was added at various times after virus inoculation and virus yields at 8 hr were compared to untreated control values (Caliguiri and Tamm, 1968b). The solid line shows that guanidine added as late as 4 hr reduces the yield of infective virus by 90%, and the broken line shows that virus RNA synthesis is reduced by 80%. This clearly establishes the fact that guanidine is effective during the exponential increase of poliovirus. The results in Fig. 7 also show that the effects of guanidine on both virus RNA synthesis and virus production diminish very rapidly after the fourth hour.

To summarize, the period of sensitivity to guanidine in the growth cycle of poliovirus begins 1 to 2 hr after infection (Eggers *et al.*, 1963b; Eggers *et al.*, 1965a,b) and continues until the fourth hour, after which it diminishes rapidly (Eggers *et al.*, 1963b; Caliguiri *et al.*, 1965; Eggers *et al.*, 1965a; Caliguiri and Tamm, 1968b). The period of guanidine sensitivity in the replication of drug-sensitive parent virus corresponds to the period during which guanidine is required in the multiplication of guanidine-dependent mutants (Eggers *et al.*, 1963b; Caliguiri *et al.*, 1965; Eggers *et al.*, 1965a,b). The period in the growth cycle of poliovirus during which guanidine acts is the period of active virus biosynthesis.

EFFECTS ON VIRUS BIOSYNTHESIS

SYNTHESIS OF VIRUS PROTEIN

After release of single-stranded poliovirus RNA from its protein coat during the early stages of virus infection, cell ribosomes attach to virus RNA to form virus polyribosomes which carry out synthesis of virus proteins (Penman *et al.*, 1963; Scharff *et al.*, 1963; Summers and Levintow, 1965). Virus RNA polymerase is made and thus virus RNA can be replicated (Baltimore *et al.*, 1963). Some of the newly made virus RNA functions in further synthesis of virus-specific proteins. Four of the 14

virus-specific polypeptides found in poliovirus-infected cells are electrophoretically identical to the four capsid proteins of the virus coat (Summers *et al.*, 1965). Summers and Maizel (1968) have shown that not all virus proteins are primary products, but that some represent cleavage products of larger precursor proteins. One of the non-capsid polypeptides is cleaved just before maturation and assembly of the complete virion (Maizel *et al.*, 1967; Jacobson and Baltimore, 1968). The function of the other virus polypeptides is not known; however, they may represent virus-specific enzymes, virus-directed inhibitors of cell biosynthesis, virus regulator proteins or merely intermediate cleavage products of precursor proteins.

Early in the investigation of the effects of guanidine on poliovirus multiplication, the use of fluorescent antibody against purified poliovirus showed that virus antigen is not formed in detectable amounts in infected cells incubated for 8 hr in the presence of guanidine (Crowther and Melnick, 1961). Similar results have been obtained by measurement of incorporation of radioactive amino-acids into virus proteins in baby hamster kidney cells infected with foot-and-mouth disease virus and treated with guanidine (Brown *et al.*, 1966). In both these studies guanidine was added at the time of infection, and thus virus protein synthesis could either be inhibited directly through a block in translation of virus RNA, or indirectly through a block in replication of virus RNA. It has been demonstrated recently that addition of guanidine to infected cells during the exponential increase phase in virus growth gives a differential effect on virus protein and RNA synthesis (Caliguiri and Tamm, 1968b). In these studies actinomycin D was added to the infected cell in order to inhibit cell RNA synthesis. Actinomycin D does not alter poliovirus RNA synthesis or virus production (Reich *et al.*, 1961). The kinetics of inhibition of virus protein and virus RNA synthesis were determined following addition of 1.5 mM guanidine 3 hr 40 min after poliovirus infection (Caliguiri and Tamm, 1968b). Figure 8 shows that 30 min after addition of guanidine the rate of virus RNA synthesis is reduced to 15% of control, whereas the rate of virus protein synthesis is still 70% of control. These results corroborate the findings of Halperen *et al.* (1964), who showed that echovirus type 12 capsid proteins continue to be formed in the presence of guanidine after virus multiplication is inhibited. It has also been shown that poliovirus capsid proteins continue to be synthesized after addition of guanidine (Jacobson and Baltimore, 1968). Taken together, the evidence indicates that the primary action of guanidine is on the replication of virus RNA and that inhibition of virus protein synthesis is a secondary effect. Clearly, interruption of virus RNA replication would

be expected to result secondarily in curtailment of virus messenger RNA available for template function in translation, and therefore in reduced synthesis of virus proteins. However, a specific block in translation of the virus RNA polymerase gene by guanidine cannot be completely excluded.

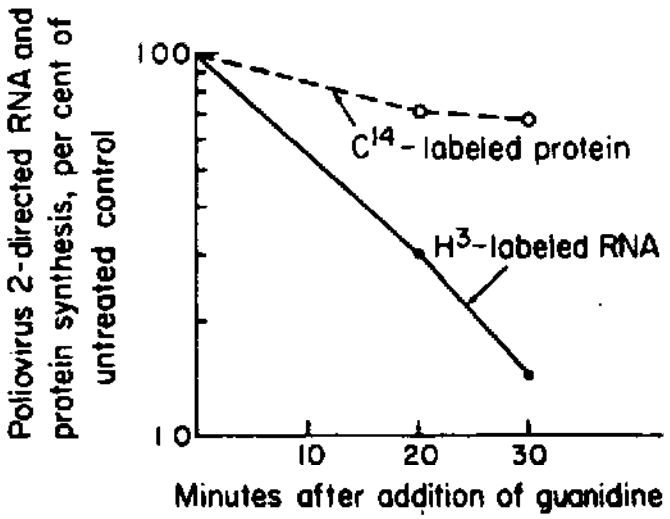

FIG. 8. Kinetics of inhibition of poliovirus RNA and protein synthesis after addition of 1.5 mM guanidine 3 hr 40 min after inoculation of actinomycin D-treated HeLa cells. (From Caliguiri and Tamm, 1968b.)

VIRUS RNA POLYMERASE

As mentioned above, poliovirus RNA polymerase is thought to be a virus-specific enzyme in that this enzyme activity is associated with the cytoplasmic fraction of infected cells and not detected in the cytoplasmic fraction of uninfected cells (Baltimore *et al.*, 1963). The activity of this enzyme can be measured *in vitro* by the incorporation of nucleoside monophosphates into RNA, but the enzyme itself has not been isolated or purified (Baltimore, 1964). Manganese ions, which stimulate the activity of cell RNA polymerase, inhibit the activity of virus RNA polymerase. Synthesis of virus RNA by this enzyme is not sensitive to actinomycin D. As already mentioned, virus RNA polymerase activity is detected in infected cells at 2 hr, after which the activity increases to a maximum at 4 hr, and then declines (Fig. 4). There is evidence from experiments with puromycin, an inhibitor of protein synthesis, that this enzyme has a short life in infected cells and that continued synthesis of enzyme is necessary for continued replication of virus RNA (Eggers *et al.*, 1963a).

Addition of guanidine at the time of infection of HeLa cells with poliovirus prevents the appearance of virus RNA polymerase activity (Baltimore *et al.*, 1963). Addition of guanidine 3 hr after infection not only prevents further increase in enzyme activity but causes a decrease in activity by 4.25 hr. These findings are compatible with rapid turnover of the enzyme. The possibility that guanidine might inactivate the enzyme has been considered and tested by addition of the chemical to the *in vitro* assay system for virus RNA polymerase. Guanidine has no demonstrable effect on the activity of virus RNA polymerase *in vitro* (Baltimore *et al.*, 1963). Similar results were obtained with guanidine and foot-and-mouth disease virus RNA polymerase (Black and Brown, 1969). When cells were infected with a guanidine-sensitive strain in the presence of guanidine, no virus RNA polymerase activity could be detected. However, guanidine had no effect when added to the *in vitro* assay system for virus RNA polymerase (Black and Brown, 1969). The *in vitro* assay system for virus RNA polymerase neither requires nor responds to added primer RNA, and therefore initiation of new RNA chains cannot be evaluated under these conditions. The lack of effect of guanidine on the *in vitro* assay for virus RNA polymerase indicates that growth of virus RNA chains can take place in the presence of guanidine; however, it leaves open the possibility that guanidine specifically blocks initiation of new RNA chains.

SYNTHESIS OF VIRUS RNA

It was pointed out above that the time courses of formation of virus RNA and capsid proteins are closely related in the poliovirus growth cycle (Fig. 4). However; these processes, transcription and translation of virus RNA, occur in association with distinct membranous structures (Caliguiri and Tamm, 1969).

Inhibition of poliovirus RNA synthesis by guanidine has been demonstrated by the use of acridine orange (Crowther and Melnick, 1961), by determination of incorporation of radioactive uridine into acid insoluble RNA (Eggers *et al.*, 1963b; Burstein *et al.*, 1966), and by autoradiography of single cells (Caliguiri *et al.*, 1965). Recently quantitative data have been obtained concerning the sensitivity of the process of virus RNA synthesis to guanidine during different periods in the virus growth cycle. Figure 9 shows that the rate of virus RNA synthesis varies during the normal growth cycle of poliovirus; in untreated poliovirus-infected cells the rate increases until the fourth hour of infection and then declines (Caliguiri and Tamm, 1968a). To determine the effect of guanidine, the inhibitor

was added to replicate cultures at different times beginning 3 hr after infection, and 10 min after addition of guanidine the rate of incorporation of uridine-^{3}H was determined. As Fig. 9 shows, guanidine has a marked inhibitory effect on the rate of virus RNA synthesis when it is added up to 4 hr after infection. The inhibitory effect progressively decreased after this time. These results clearly establish that the inhibitory effect of guanidine is related to the phases of virus RNA synthesis.

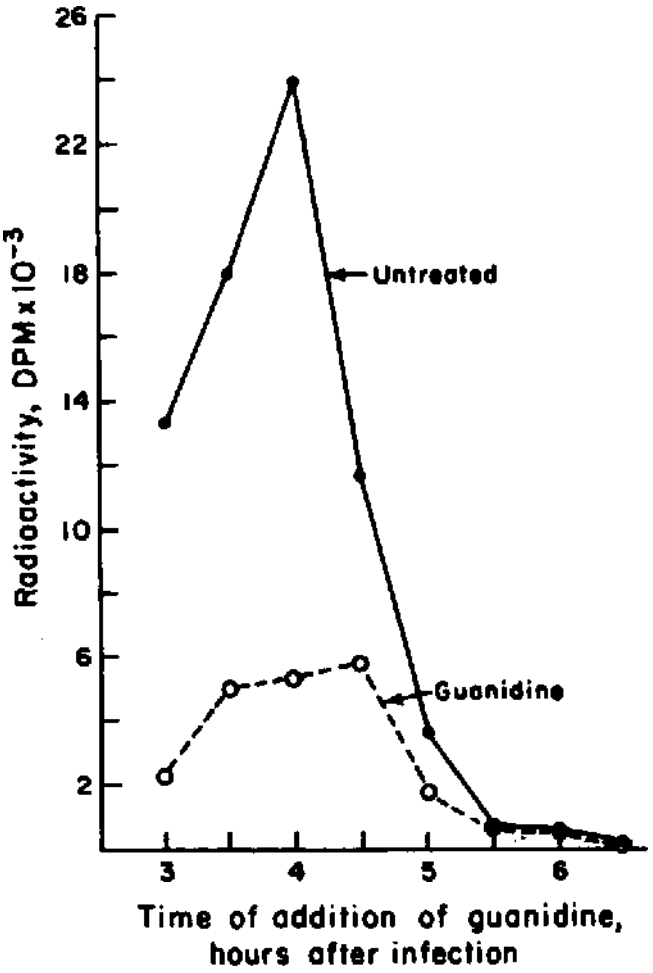

FIG. 9. Rate of poliovirus RNA synthesis and inhibition in actinomycin D-treated HeLa cells 10 min after addition of 1.5 mM guanidine at various times after inoculation. (From Caliguiri and Tamm, 1968a.)

Of special interest are the findings concerning the effects of guanidine on the formation of the three known virus-specific species of RNA found in the infected cells. Figure 10A shows the sedimentation pattern of pulse-labeled poliovirus-specific RNA in sucrose density gradients. The major

peak of virus RNA is single-stranded and sediments at 35S. Single-stranded 35S RNA is the species of RNA found in the mature poliovirus particle (Holland *et al.*, 1960; Darnell, 1962). RNA sedimenting in the 16–20S region is most resistant to ribonuclease digestion and represents the double-stranded replicative form of poliovirus RNA. The RNA which sediments

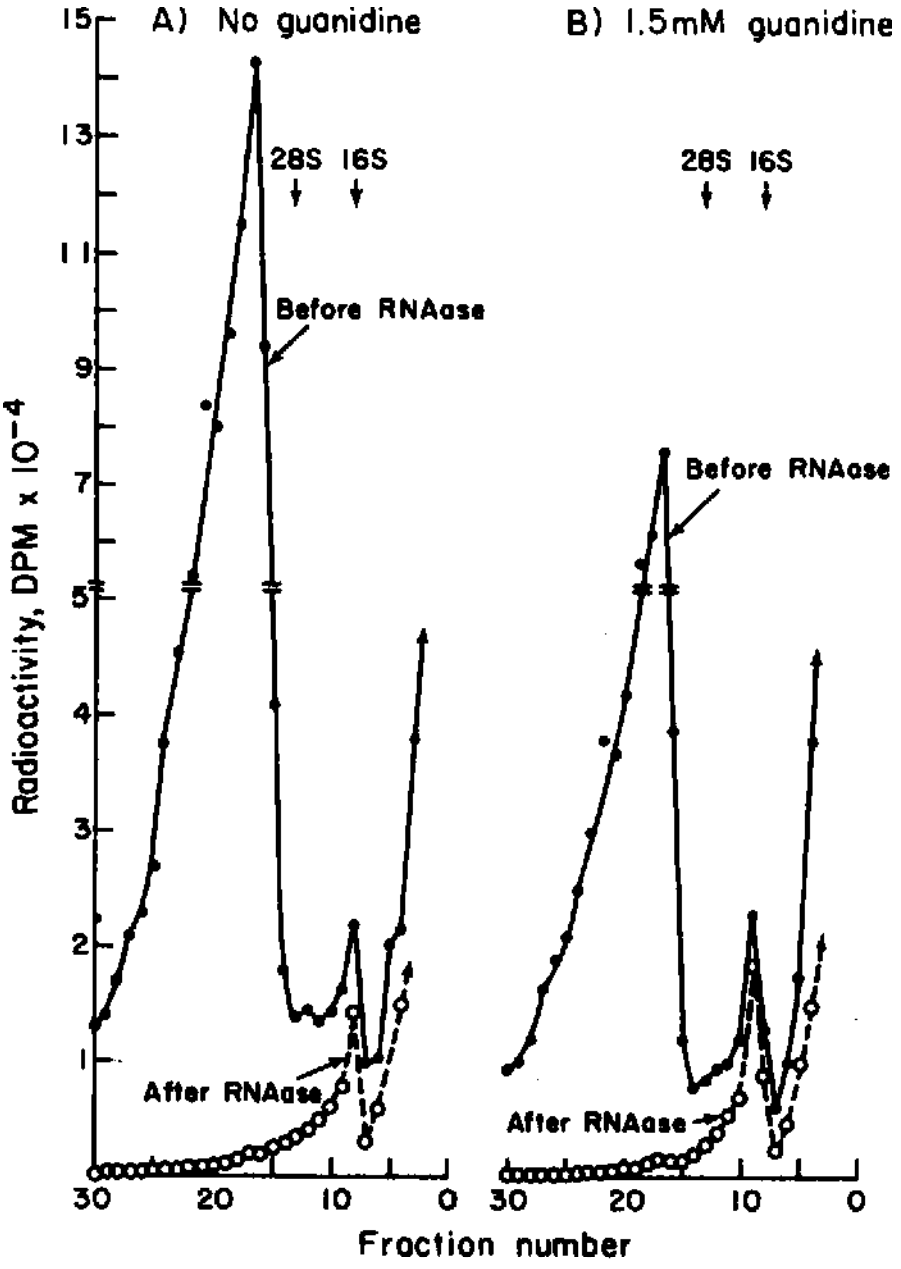

FIG. 10. Sedimentation analysis of poliovirus-specific species of RNA pulse-labeled 3.25 hr after inoculation in the presence or absence of 1.5 mM guanidine. The cultures were treated with actinomycin D prior to pulse labeling. (From Caliguiri and Tamm, 1968a.)

faster than 20S and shows a lesser degree of resistance to ribonuclease probably represents the multistranded replicative intermediate species of poliovirus RNA (Baltimore and Girard, 1966).

The sedimentation pattern in Fig. 10B shows the virus-specific RNA pulse-labeled during the first 12.5 min after addition of guanidine. In guanidine-treated cells synthesis of virus-specific RNAs is markedly reduced; however, single-stranded 35S RNA is the major RNA moiety formed. The amount of labeled RNA which sediments faster than 20S is reduced in the presence of guanidine, whereas the amount of ribonuclease-resistant RNA in the 16–20S region is unaffected. Similar results have been obtained by separation of cumulatively labeled double-stranded RNA from single-stranded RNA and replicative intermediate on the basis of differential solubility in M NaCl (Caliguiri and Tamm, 1968a). It may be concluded that in guanidine-treated cells there is a net reduction in the single-stranded RNA and multistranded replicative intermediate, whereas the amount of double-stranded ribonuclease-resistant RNA continues to increase. The decrease in multistranded replicative intermediate suggests that nascent RNA chains are completed, but new RNA chains are not initiated in the presence of guanidine. The increase in double-stranded RNA probably represents template devoid of nascent RNA since completion occurs without initiation of new RNA chains.

It was of interest to determine if RNA chains completed in the presence of guanidine could be incorporated into virus polyribosomes. The findings indicate that guanidine does not interfere with the incorporation of progeny virus RNA into polyribosomes (Caliguiri and Tamm, 1968b). In these experiments EDTA was used to disperse polyribosomes and remove pulse-labeled RNA from the polyribosomal region of sucrose density gradients. In cytoplasmic extracts from untreated cells (Fig. 11A) a major part of the broad peak of pulse-labeled RNA in the sucrose-RSB gradients was removed by EDTA. The broad band of RNA that remained in the heavier region of the sucrose-EDTA gradient is probably associated with the replication complex. The replication complex is associated with smooth membranes, and after deoxycholate treatment it sediments heterogeneously (Caliguiri and Tamm, 1969). Although the nature of this structure is poorly understood, virus RNA polymerase and virus RNA labeled after a short pulse are associated with the replication complex. In the extracts from cells treated with guanidine 10 min before pulse-labeling the RNA, the amount of RNA in the lower half of sucrose-RSB gradients is markedly reduced (Fig. 11B). All of the RNA in this region is removed by EDTA. These results suggest that, after 10 min of treatment of infected cells with

guanidine, newly formed virus RNA can still be incorporated into virus polyribosomes and there is no detectable RNA in the replication complex (Caliguiri and Tamm, 1968b).

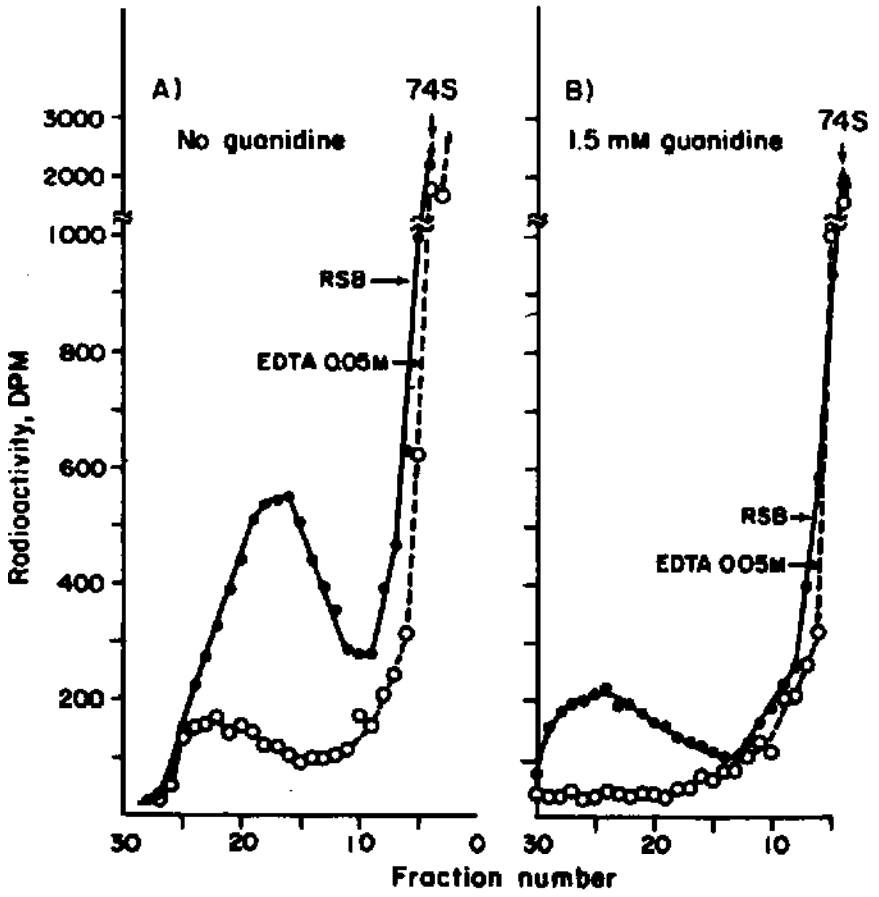

FIG. 11. Effects of 0.05 M EDTA on poliovirus RNA pulse-labeled in the presence or absence of 1.5 mM guanidine. The cultures were treated with actinomycin D prior to pulse-labeling. (From Caliguiri and Tamm, 1968b.)

FORMATION OF VIRUS PARTICLES

Kinetic experiments show that guanidine causes complete inhibition of virus production within 1 hr of addition to infected cells; however, during the first 30–60 min there is some production of infective virus (Crowther and Melnick, 1961; Eggers *et al.*, 1963b; Caliguiri *et al.*, 1965). Figure 12 shows a 3-fold increase in infective virus during the first 30-min interval after addition of guanidine at 3 hr (Caliguiri and Tamm, 1968b). Figure 13 shows that virus RNA synthesized in the presence of guanidine can be incorporated into virus particles. In this experiment the cultures received

a pulse of uridine-^{3}H 10 min after addition of guanidine and the cytoplasmic extracts were incubated with ribonuclease before sedimentation to digest the RNA not enclosed by virus coat protein. The peak of ribonuclease-resistant labeled RNA coincides with the peak of infectivity,

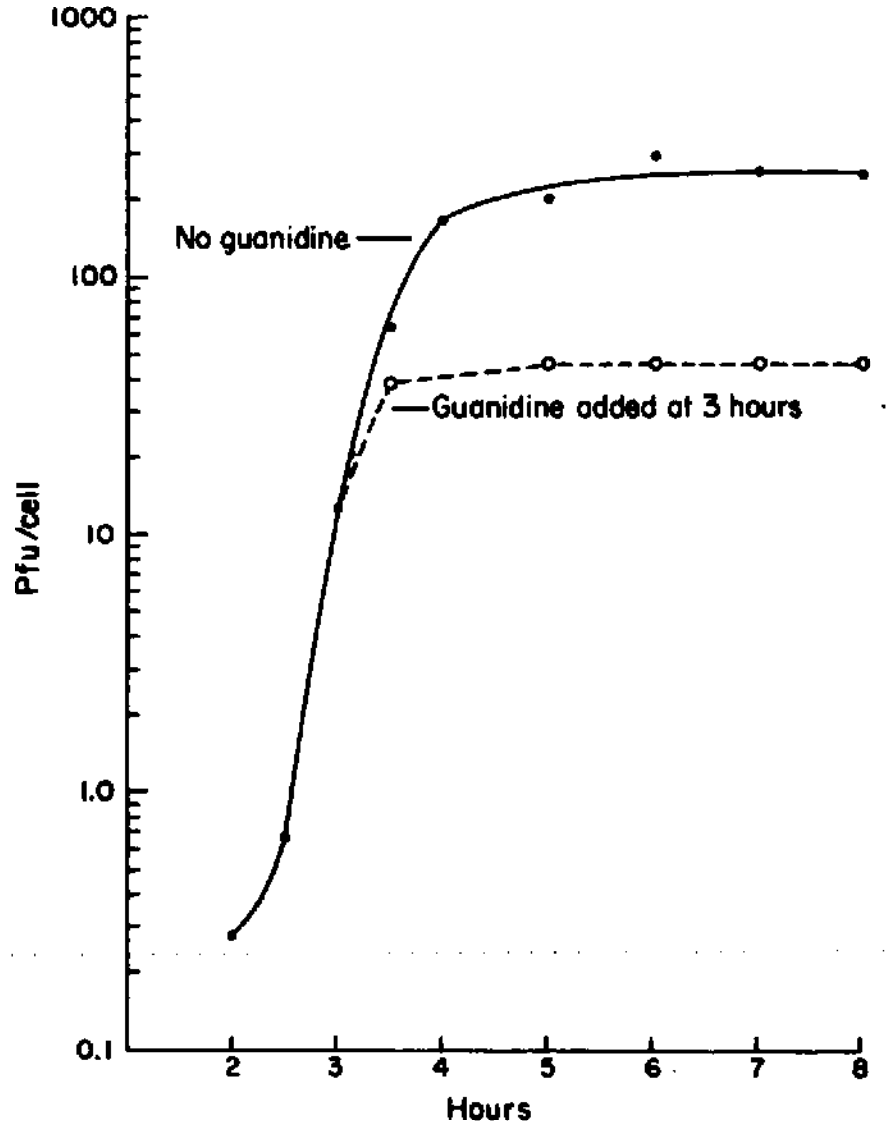

FIG. 12. Effects of 1.5 mM guanidine on the formation of infective poliovirus. (From Caliguiri and Tamm, 1968b.)

and represents RNA incorporated into mature virus particles. The available evidence suggests that the effect of guanidine on production of infective virus is secondary to its effect on synthesis of virus RNA.

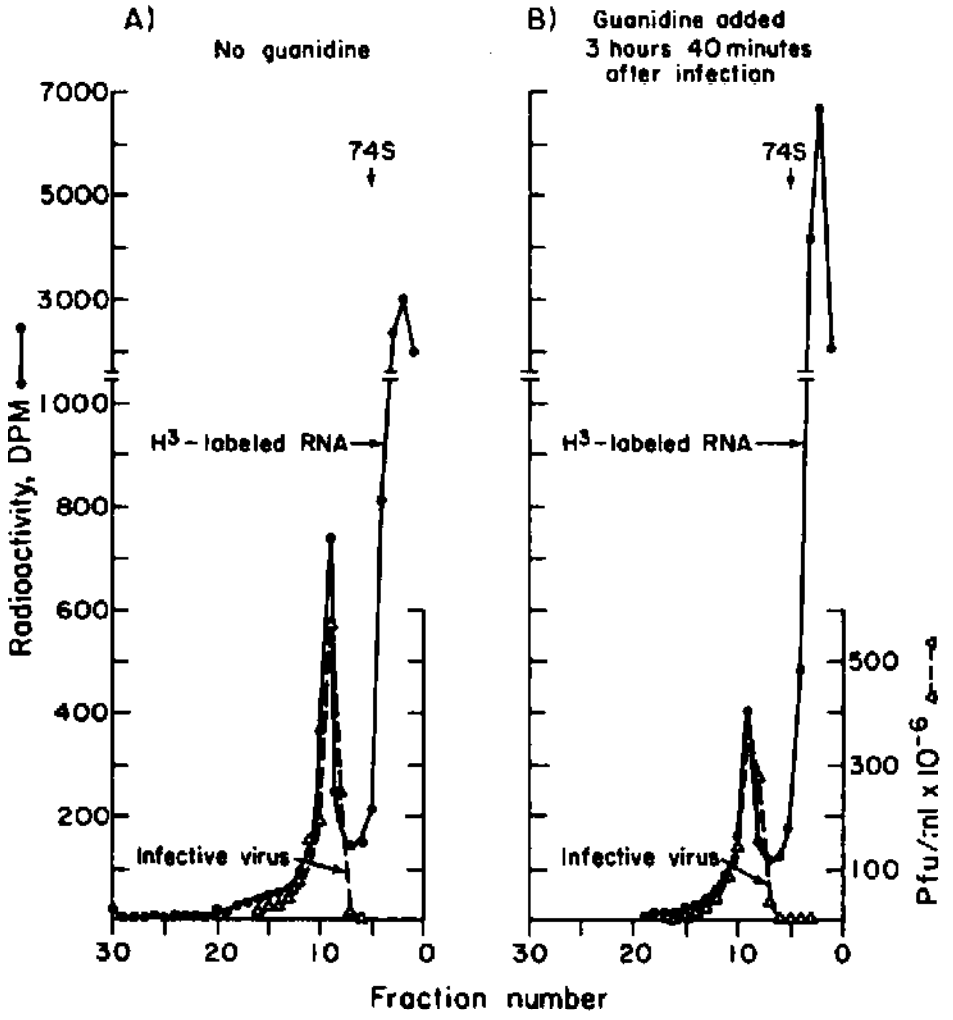

FIG. 13. Effects of 1.5 mM guanidine on the incorporation of pulse-labeled poliovirus RNA into mature virus particles. (From Caliguiri and Tamm, 1968b.)

THEORIES OF MECHANISM OF ACTION

Kinetic studies have clearly defined the period during the virus growth cycle when guanidine is effective. The period of sensitivity to guanidine begins between 1 and 2 hr after inoculation and declines after the fourth hour. Analysis of the events which take place at various times in the growth cycle relate the effectiveness of guanidine to biosynthesis of virus products. The initial events of virus infection such as adsorption, penetration, or virus-induced inhibition of cell biosynthesis are not altered significantly in the presence of guanidine. The period of sensitivity to guanidine coincides with the period of virus biosynthesis in which translation and transcription of virus RNA takes place. The later events such as assembly and release of mature virus are probably not affected by

guanidine directly. However, any mechanism which restricts synthesis of virus structural proteins and virus RNA will rapidly restrict production of virus particles. Since all the results indicate that virus biosynthesis is the critical process which is inhibited by guanidine, only those theories which relate the mechanism of action of guanidine to virus biosynthesis will be discussed.

In picornavirus-infected cells, translation and replication of virus RNA are closely related temporally in the cell. The onset of these processes is interdependent and both processes are associated with membranous structures in the cytoplasm of the cell. It has been shown that guanidine inhibits replication of virus RNA much more rapidly than translation of virus RNA, i.e. virus protein synthesis. Furthermore, synthesis of coat proteins and the formation of empty capsids do occur in the presence of guanidine after virus RNA synthesis has been inhibited. This suggests that guanidine directly inhibits virus RNA replication.

The onset of virus RNA replication and virus RNA translation are interdependent, since virus RNA replication requires the synthesis of a virus-directed enzyme, virus RNA polymerase. This enzyme is labile and continued synthesis of virus RNA polymerase is necessary for continued virus RNA synthesis. Guanidine prevents the appearance of this enzyme in infected cells and causes rapid reduction in this enzyme after synthesis has already started; however, guanidine has no effect on the activity of virus RNA polymerase when added to the *in vitro* assay system for this enzyme. The initial interpretation of these results was that guanidine inhibits synthesis of virus RNA polymerase but does not alter its function. However, there is another mechanism which now appears more likely to explain the reduction in virus RNA polymerase activity after treatment of infected cells with guanidine. So far poliovirus RNA polymerase has been extracted only as an aggregate enzyme which consists of template RNA in association with the enzyme. This enzyme preparation does not respond to, nor require, added primer RNA, and therefore initiation of RNA synthesis cannot be investigated specifically. Inhibition of initiation of virus RNA chains by guanidine would soon deplete virus RNA polymerase of RNA template in infected cells. The aggregate enzyme devoid of RNA template would not be active when assayed *in vitro*. The fact that guanidine has no effect on virus RNA polymerase activity when added to the *in vitro* assay system indicates that guanidine permits the growth of previously initiated RNA chains but leaves open the possibility that guanidine may inhibit RNA chain initiation.

The strongest evidence that guanidine blocks initiation of virus RNA

chains is the observation that treatment of infected cells with guanidine results in a decrease in the amount of replicative intermediate species of virus RNA. If the replicative intermediate is the true precursor form of virus RNA, a net reduction in this species of RNA indicates that guanidine blocks initiation of new RNA chains and allows previously initiated chains to be completed. Further evidence in support of this view is the absence of detectable nascent RNA in the replication complex 10 min after addition of guanidine. The degree of sensitivity to guanidine during virus growth can also be related to synthesis of virus RNA. Guanidine is highly active early in the growth cycle when many new virus RNA chains are initiated, but it becomes progressively less so after the fourth hour when probably few new chains are initiated.

It has been shown that virus RNA synthesized 10 min after addition of guanidine can be incorporated into polyribosomes and virus particles. The distribution of newly formed virus RNA into these structures suggests that such RNA is released from the replication complex in the presence of guanidine. The effect of guanidine on production of infective poliovirus is probably secondary to its effect on synthesis of virus RNA. Newly made RNA is normally incorporated into virus particles within a few minutes of its synthesis, so that any restriction on virus RNA replication will rapidly restrict production of infectious virus particles. However, this does not exclude the possibility of a direct effect of guanidine on virus maturation, which would be detectable only if assembly of virus were to occur from preformed virus precursors.

The evidence obtained so far supports the view that initiation of virus RNA replication is the process inhibited by guanidine. Initiation of virus RNA replication is a complex process which requires the interaction of template RNA, virus RNA polymerase, cell membranes, and possibly other factors which have not been defined as yet. It has been shown that guanidine-sensitive virus can replicate its own RNA in guanidine-treated cells which are coinfected with a guanidine-dependent mutant (*vide infra*). The fact that the RNA of the sensitive virus can replicate under these conditions, presumably through the RNA polymerase provided by the guanidine-dependent virus, suggests that guanidine does not interact with template RNA to block initiation.

Interaction between virus RNA polymerase and guanidine is more likely and might result in allosteric inhibition of the sensitive enzyme. Lwoff suggested in 1965 that guanidine might interfere with the morphogenesis of active enzyme from protein subunits. This would reduce the number of new active polymerase molecules and inhibit the initiation of

new virus RNA chains. Alternatively, guanidine may interact with fully formed virus RNA polymerase and block initiation of RNA chains. This hypothesis predicts that drug-resistant mutants synthesize a virus RNA polymerase whose functional conformation is not altered by guanidine. Furthermore, the functional conformation of the enzyme synthesized by the drug-dependent virus may be induced by guanidine.

If there are two virus RNA polymerases, one which catalyzes the synthesis of minus strands of RNA and another which catalyzes the synthesis of plus strands of RNA, only one of the enzymes might interact with guanidine. The evidence suggests that synthesis of the minus strand and formation of the replicative form occurs in the presence of guanidine, whereas the synthesis of plus strands is blocked.

The function of the cell membranes to which the replication complex is attached has not been fully investigated as yet. Investigation may reveal that membranes play a critical role in this process. Guanidine may block the normal interaction between the replication complex and membranes which could inhibit initiation of virus RNA chains. Another possibility is that guanidine reacts with some as yet unidentified virus-specific component which affects initiation of virus RNA chains.

Compounds such as methionine and choline which are capable of partially reversing inhibition of poliovirus growth by guanidine have not been investigated thoroughly enough to relate them to the mechanism of action of guanidine. However, a more complete knowledge of guanidine antagonists should further advance our understanding of the site of action of guanidine.

EFFECTS ON VIRUS-INDUCED ALTERATIONS IN CELLS

VIRUS-INDUCED INHIBITION OF CELL BIOSYNTHESIS

Cell polyribosomes disperse between 1 and 2 hr after infection of cells with poliovirus (Penman *et al.*, 1963), and there is inhibition of cell protein (Zimmerman *et al.*, 1963; Holland and Peterson, 1964), RNA (Zimmerman *et al.*, 1963; Holland, 1963; Bablanian *et al.*, 1965a) and DNA synthesis (Salzman *et al.*, 1959; Holland and Peterson, 1964). The mechanisms by which poliovirus inhibits these cell functions are not well understood; however, these inhibitions appear to be mediated through newly synthesized virus products. The findings which suggest that synthesis of virus products is necessary are as follows: there is a delay in the onset of virus-induced inhibition which corresponds to the period of exposure to

inhibitors of protein synthesis (Penman and Summers, 1965); neither UV-inactivated (Penman and Summers, 1965) nor photodynamically inactivated poliovirus (Holland, 1964) inhibit cell biosynthesis when added to cells; purified empty capsids of poliovirus do not inhibit HeLa cell DNA or protein synthesis (Ensminger and Caliguiri, unpublished data).

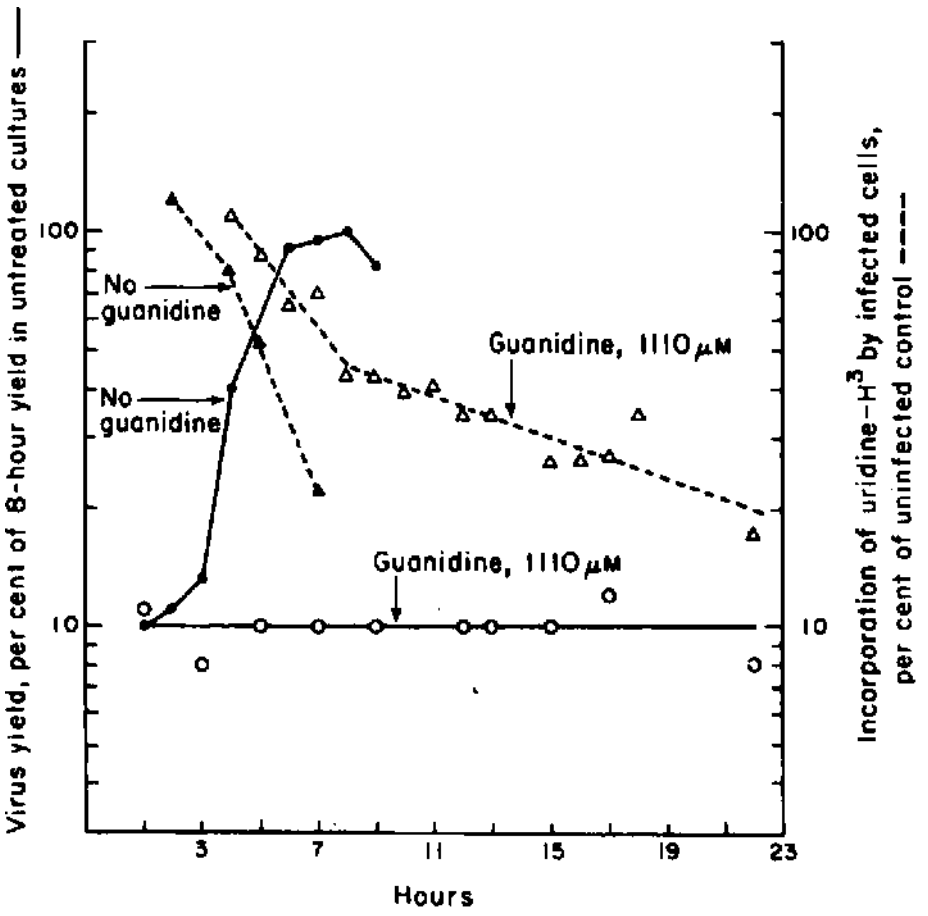

FIG. 14. Effects of guanidine on the rate of RNA synthesis in poliovirus-infected human embryonic lung cells and on virus yield. (From Bablanian, Eggers and Tamm, 1965a.)

Guanidine does not prevent virus-induced inhibition of cell biosynthesis in cells that are infected at multiplicities of 100 plaque-forming units (PFU) per cell or more (Bablanian *et al.*, 1965a; Penman and Summers, 1965). Figures 14 and 15 illustrate the effects of guanidine on virus-induced inhibition of protein and RNA synthesis. In these experiments the rate of RNA synthesis (Fig. 14) and the rate of protein synthesis (Fig. 15) were

determined by pulse-labeling guanidine-treated and untreated cultures with radioisotopes at various times after infection. The data indicate that in the presence of 1.11 mM guanidine, virus-induced inhibition of cell protein and RNA synthesis is delayed but not prevented (Bablanian *et al.*, 1965a). These results have been corroborated in experiments with poliovirus-infected cells in which cumulative incorporation of radioisotopes

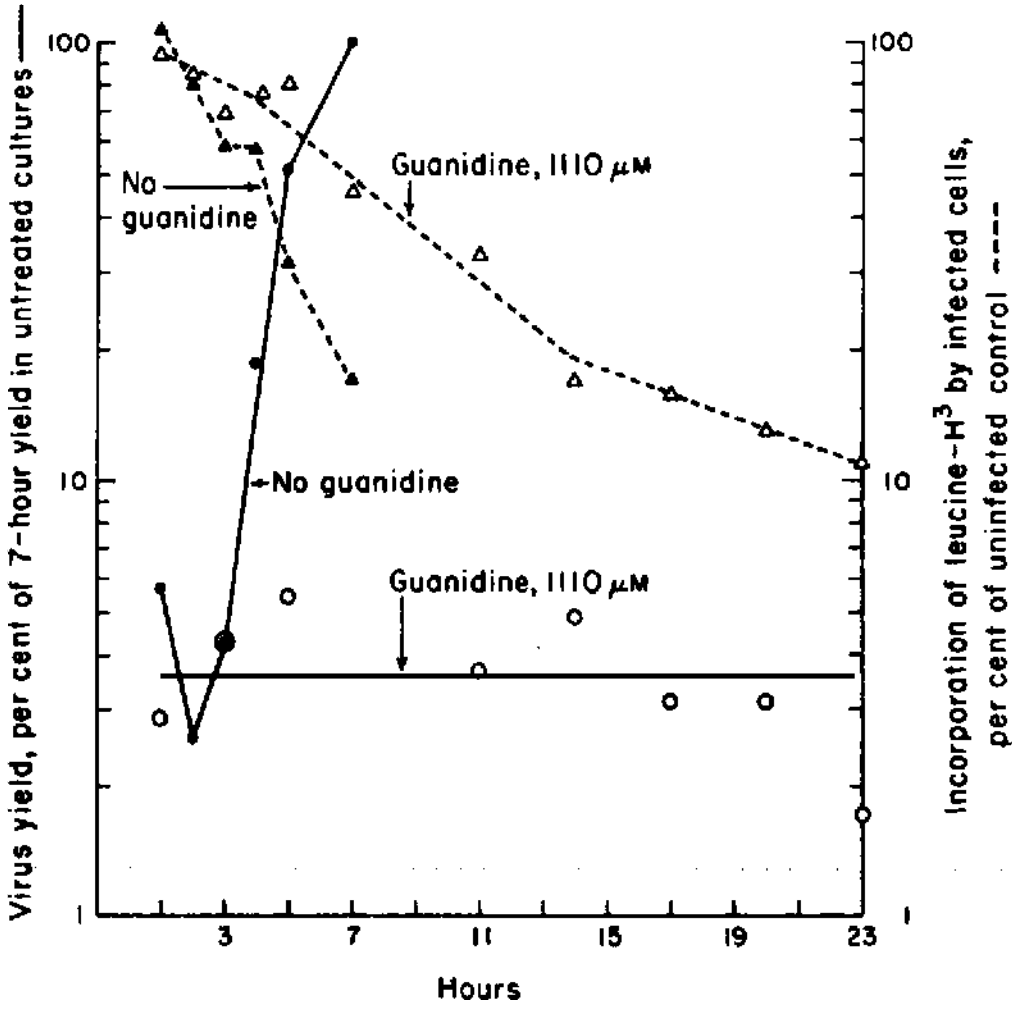

FIG. 15. Effects of guanidine on the rate of protein synthesis in poliovirus-infected human embryonic lung cells and on virus yield. (From Bablanian, Eggers and Tamm, 1965a.)

into protein and RNA was measured in the presence or absence of guanidine (Penman and Summers, 1965). In addition, 2.5 mM guanidine does not prevent inhibition of biosynthesis in cells infected at the very high multiplicity of 10,000 PFU/cell (Holland, 1964). However, it has

been reported that 2.5 mM guanidine does prevent virus-induced inhibition of cell biosynthesis in cells infected at a low multiplicity of 10 PFU/cell (Holland, 1964). As discussed below, this discrepancy can be explained on the basis of messenger capabilities of varying numbers of virus RNA molecules introduced at infection.

The evidence suggests that synthesis of early virus-directed products is responsible for inhibition of cell biosynthesis and that guanidine does not prevent the synthesis of these products. The rates at which these products accumulate in infected cells would be expected to depend both on the amount of input virus RNA and the amount of *de novo* synthesized virus RNA. The delay in the development of virus-induced inhibition in guanidine-treated cells infected at a multiplicity of 100 PFU/cell may be explained as follows: Since guanidine prevents the synthesis of new virus RNA, the accumulation of inhibitory products is entirely dependent on the messenger function of the input virus RNA. Thus, time may be required for quantities of inhibitory products to be synthesized which are sufficient to inhibit cell biosynthesis.

After infection of cells at even lower multiplicities, such as 10 PFU/cell, the input RNA may be insufficient to direct the synthesis of enough inhibitory products to produce demonstrable inhibition of cell biosynthesis; here guanidine would be expected to prevent completely virus-induced inhibition of cell biosynthesis. Conversely, infection of cells at 10,000 PFU/cell may introduce a quantity of virus RNA sufficient to make inhibitory products at rates assuring maximal inhibition of cell biosynthesis; here guanidine would not be expected to have any effect. This interpretation has been suggested previously (Bablanian *et al.*, 1965a), but confirmation of this hypothesis must await further clarification of the mechanism by which virus infection inhibits cell biosynthesis.

EFFECTS ON VIRUS-INDUCED MORPHOLOGICAL CELL DAMAGE

The time course of morphological cell damage in poliovirus-infected cells is related to the production of infective virus in Fig. 16. Cell damage is first detected between 4 and 4.5 hr after infection, a time when production of infective virus is increasing exponentially (Bablanian *et al.*, 1965b). Addition of guanidine at the time of infection or any time up to 2 hr after infection prevents the development of virus-induced cell damage during a single cycle of virus growth (Fig. 17) (Bablanian *et al.*, 1965b). In view of the fact that guanidine does not prevent virus-induced inhibition of

cell biosynthesis (Bablanian *et al.*, 1965a,b), it is unlikely that virus-induced morphological changes are a direct consequence of virus-directed inhibition of cell biosynthesis (Martin and Work, 1961; Holland, 1963; Holland, 1964).

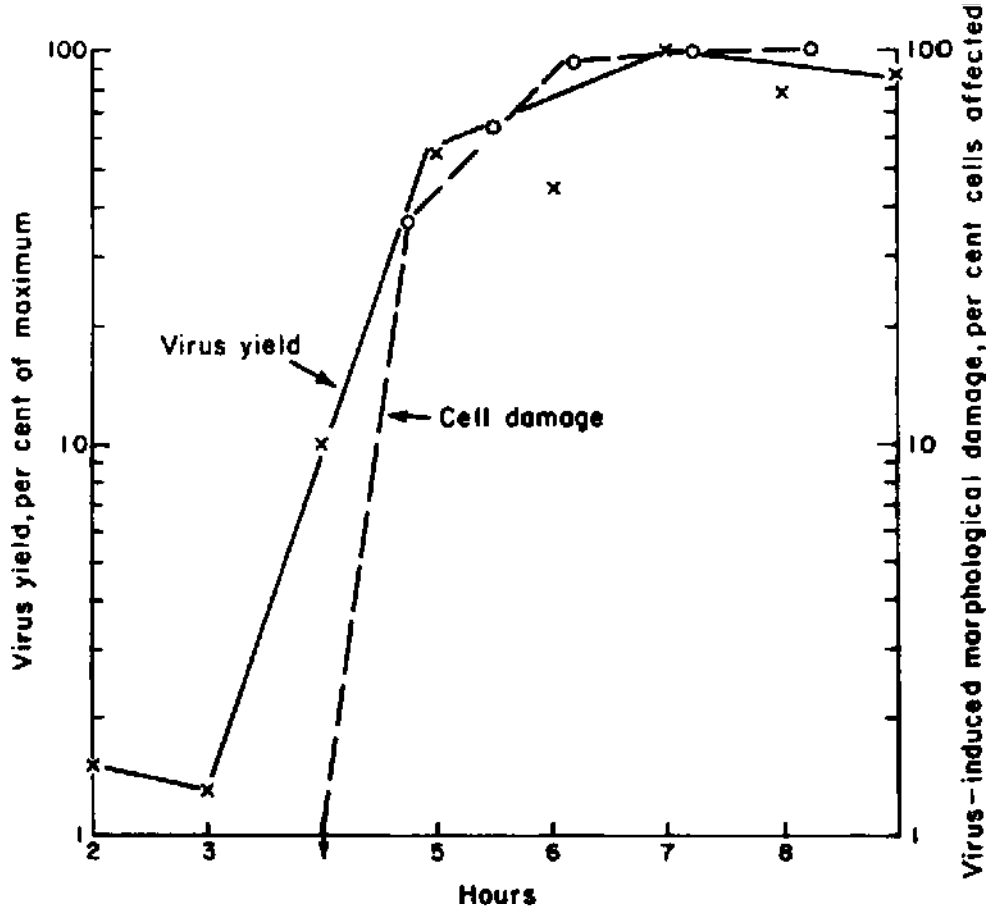

FIG. 16. Time course of the multiplication of poliovirus and the development of virus-induced cell damage. (From Bablanian, Eggers and Tamm, 1965b.)

The effectiveness of guanidine in preventing virus-induced cytopathic changes diminishes when the inhibitor is added after 2 hr. Guanidine added at 4 hr after infection does not prevent virus-induced cell damage (Fig. 17), although virus yield is still reduced by 90% (Fig. 7) (Caliguiri and Tamm, 1968b). These data indicate that maximal virus yields are not necessary for maximal cell damage.

When guanidine is added later than 2 hr after infection, it does not prevent virus-induced cell damage (Bablanian *et al.*, 1965b). This suggests that the synthesis of virus protein(s) responsible for virus-induced cell

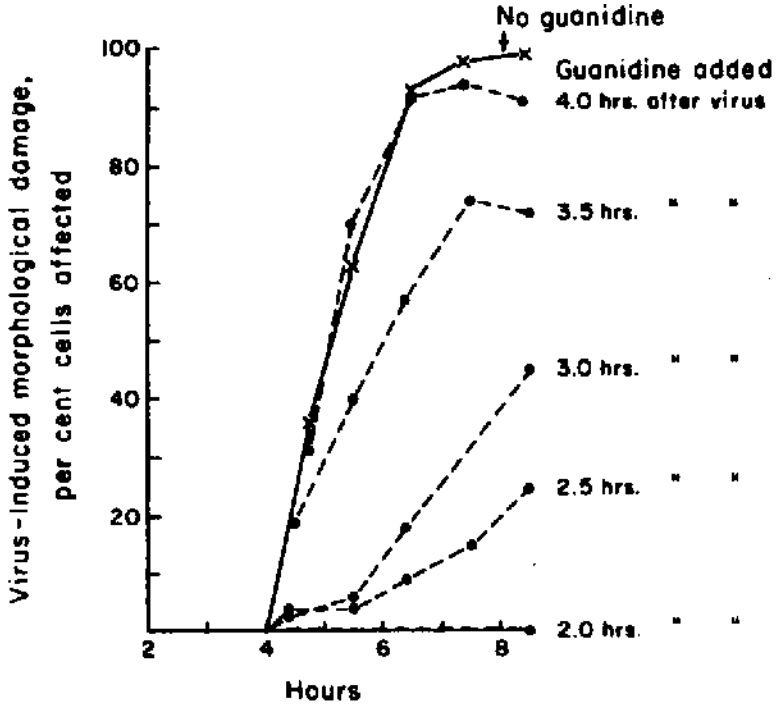

FIG. 17. Relation between time of addition of 1.1 mM guanidine and inhibition of the development of poliovirus-induced cell damage. (From Bablanian, Eggers and Tamm, 1965b.)

changes begins 2 hr after infection. This idea was substantiated through a comparison of the effects of guanidine and an inhibitor of protein synthesis, puromycin, on virus-induced cell damage (Fig. 18). When virus yield is measured, the addition of either guanidine or puromycin at various times after infection has similar inhibitory effects on virus production (Fig. 18a) (Bablanian *et al.*, 1965b). In contrast, addition of guanidine at 3.5 hr after infection does not prevent virus-induced cytopathic effects; 25 μM puromycin added at this time reduces such changes and 200 μM puromycin protects 90% of the cells from virus-induced damage (Fig. 18b). This is strong evidence in favor of the hypothesis that virus-induced morphological changes are brought about by virus-directed proteins and that guanidine does not prevent synthesis of these proteins.

DRUG RESISTANCE AND DRUG DEPENDENCE

Historically, the development of drug-resistant organisms was recognized shortly after experimental chemotherapy was first established by Ehrlich (Work and Work, 1948). Workers in Ehrlich's laboratory reported

the emergence of drug-resistant trypanosomes in 1902. This clearly establishes the problem of drug resistance to be as old as that of chemotherapy. It is noteworthy that even with the small and simple picornaviruses, guanidine-resistant mutants were reported soon after the antiviral activity of guanidine was discovered (Melnick *et al.*, 1961). This report was followed by others in which mutants dependent on the presence of guanidine for replication were reported along with mutants resistant to the drug.

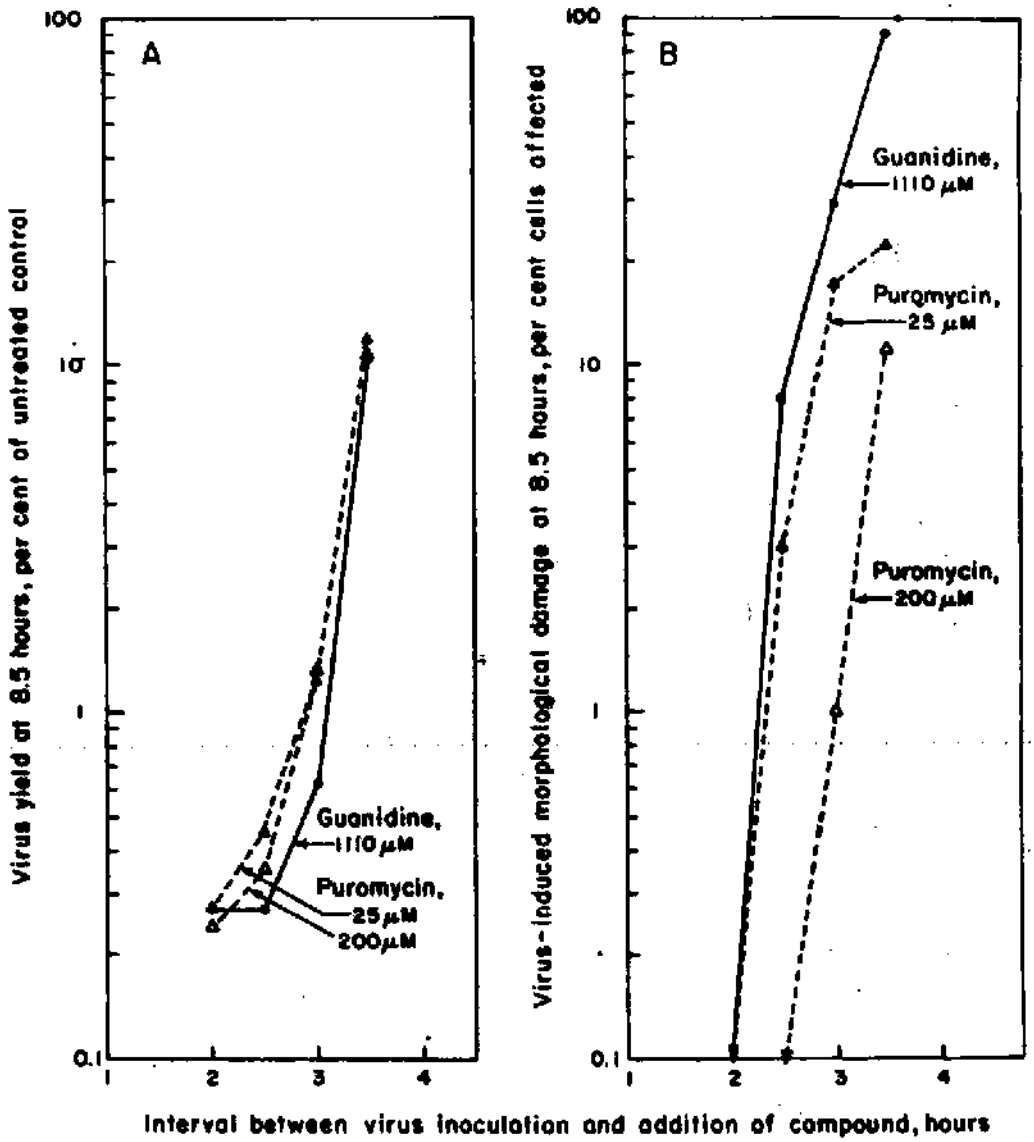

FIG. 18. Relation between time of addition of guanidine or puromycin and extent of inhibition of poliovirus multiplication and virus-induced cell damage. (From Bablanian, Eggers and Tamm, 1965b.)

Resistance to guanidine develops in a pattern similar to that found in the development of bacterial resistance to streptomycin in that mutants resistant to a range of concentrations of guanidine can be isolated after a single exposure of poliovirus to guanidine. For example, Ledinko (1963) found mutants resistant to guanidine at concentrations from 27 μg/ml to 60 μg/ml after poliovirus was grown in the presence of 40 μg/ml of guanidine. Repeated passage of poliovirus in the presence of high concentrations of guanidine results in the emergence of mutants that require guanidine for normal replication (Loddo *et al.*, 1962b; Lwoff *et al.*, 1963a). None of the data currently available distinguish clearly between single or multiple mutational sites for guanidine resistance. It is possible that further genetic analysis may reveal the presence of suppressor mutations (Cooper, 1968) similar to those found associated with streptomycin resistance in *E. coli* (Gorini and Beckwith, 1966).

Several other aspects of guanidine resistance have emerged from investigations of this genetic character in poliovirus. Hydroxylamine has been used successfully to induce guanidine resistance in poliovirus (Klein and Teodorescu, 1968). Ueda and co-workers (1963b) reported that guanidine-resistant progeny poliovirus did not appear until 34 hr after inoculation of HeLa cells at a multiplicity of 20 PFU/cell in the presence of 1 mM guanidine. The emergence of guanidine-resistant mutants with time is similar to the break-through which occurs in the presence of HBB (see preceding chapter). Most plaques produced in the presence of guanidine are smaller than the wild type plaques when first isolated (Ledinko, 1963; Nakano *et al.*, 1963); however on further passage in the presence of guanidine the plaque size becomes similar to that characteristic of wild type virus. The growth kinetics of the resistant mutants are similar to those of guanidine-sensitive virus (Eggers *et al.*, 1965a). Lwoff (Lwoff and Lwoff, 1964a) found that although glutamine, semicarbazide and agmatine do not inhibit guanidine-sensitive poliovirus, these compounds do support the growth of guanidine-dependent mutants. The reversion rates for both the guanidine-resistant (Ledinko, 1963; Cooper, 1968) and guanidine-dependent (Nakano *et al.*, 1963) mutants are rather high. Ledinko (1963) has found that as much as 0.05% of a guanidine-dependent mutant population does not require guanidine for growth.

Extensive kinetic studies have been carried out to establish the drug-requiring period for the normal development of guanidine-dependent poliovirus (Eggers *et al.*, 1963b; Eggers *et al.*, 1965a; Caliguiri *et al.*, 1965). Guanidine is required beginning 1 hr after infection until late into the exponential increase phase of virus growth. This period corresponds

exactly to the period when the growth of wild type poliovirus is sensitive to guanidine. The appearance of virus RNA polymerase activity in guanidine-dependent poliovirus-infected cells also requires the presence of guanidine (Baltimore *et al.*, 1963). As expected, guanidine sensitivity or guanidine dependence is a property of the virus RNA. This has been shown by phenol extraction of RNA from guanidine-sensitive and guanidine-dependent poliovirus and determination of the infectivity of the extracted RNA in the presence or absence of guanidine (Loddo *et al.*, 1963b; Carp, 1964). RNA from guanidine-dependent virus is only infective in the presence of guanidine while the reverse is true for RNA from guanidine-sensitive virus. All the evidence suggests that the drug-requiring process in the multiplication of dependent virus is analogous to the drug-sensitive process in the multiplication of sensitive virus (Eggers *et al.*, 1965a).

The emergence of drug-resistant and drug-dependent mutants has led to genetic studies which will be discussed in the following section. These mutants have been useful in establishing a genetic map for the poliovirus genome and have also increased our understanding of the action of guanidine.

GENETIC STUDIES

Genetic studies of poliovirus present problems common to all small RNA viruses: a high rate of mutation and a high rate of reversion. However, two main types of genetic experiments have contributed to our understanding of the genetic character of poliovirus and the biochemical basis of guanidine sensitivity. Results of recombination experiments suggest the possible locus of guanidine resistance on the poliovirus genome (Ledinko, 1963; Cooper, 1968). Rescue experiments utilizing guanidine-dependent mutants and guanidine-sensitive wild type virus have thrown light on the biochemical relationship between these viruses (Cords and Holland, 1964; Ikegami *et al.*, 1964; Wecker and Lederhilger, 1964).

RECOMBINATION

Ledinko (1963) first determined the recombination frequency for a variety of guanidine-resistant mutants by crosses with horse serum-resistant mutants. The results of these experiments show that mutants with widely differing degrees of resistance to guanidine have the same frequency of recombination. Regardless of which guanidine-resistant

mutants are used for mixed infection, the yield of recombinants is about 0.4%. Ledinko (1963) also found that the recombination frequency is the same with an input multiplicity between 10 and 30 PFU per cell as long as the input ratio between mutants is between 0.5 and 3. These results have been confirmed by Cooper (1968) in a study in which temperature-sensitive and guanidine-resistant mutants were used to obtain a genetic map on the basis of recombination frequencies.

Isolation of mutants with many intermediate degrees of resistance to guanidine suggests that there may be multiple sites for guanidine resistance on the poliovirus genome. Ledinko (1963) noted, however, that recombinants of intermediate resistance were not obtained and that the low resolution of the system used may not permit detection of such intermediates. Cooper (1968) was able to map guanidine resistance as a single locus on the poliovirus genome by selecting mutants with additive recombination frequencies. Three-factor crosses were performed utilizing a single temperature-sensitive guanidine-resistant mutant and three other temperature-sensitive mutants. Many of the mutants tested were found to behave as double mutants and therefore could not be used for this purpose. Cooper (1968) also noted that the revertant character was not reliable because of probable suppressor mutations. The presence of suppressor mutations in bacterial genetics is well documented and has been particularly useful in understanding streptomycin resistance (cf. review by Gorini and Beckwith, 1966). Although suppressor mutations have not been demonstrated for poliovirus, this possibility must be considered when explaining the genetic behavior of guanidine resistance.

RESCUE

Cells which are doubly infected with guanidine-sensitive and guanidine-dependent poliovirus in the presence or absence of guanidine yield progeny virus of both types, guanidine-dependent and guanidine-sensitive. A number of investigators have reported that guanidine-dependent poliovirus can be rescued by guanidine-sensitive poliovirus in the absence of guanidine and that rescue of guanidine-sensitive by guanidine-dependent virus in the presence of guanidine also occurs (Holland and Cords, 1964; Ikegami *et al.*, 1964; Wecker and Lederhilger, 1964; Agol and Shirman, 1964). Although the yield of rescued virus is rather low, the genotype of the rescued virus is the same as that of the parent virus in regard to guanidine dependence. All the progeny isolated from the mixed infection possess the same antigenic coat protein, which is that of the assisting virus. Similar

results are obtained when infective RNA from dependent and sensitive virus is used in place of whole virus particles (Cords and Holland, 1964; Ikegami *et al.*, 1964).

These results show that drug-dependent mutants can replicate under restrictive conditions if a non-restricted virus is present in the infected cell. Since virus RNA synthesis is inhibited under restrictive conditions, initiation of virus RNA replication of the rescued virus is thought to occur through conditions brought about by the assisting virus. If the same enzyme functions in the replication of both virus RNAs, the replication of RNA of the rescued virus appears to be rather inefficient, since the yield of rescued virus is low. Inefficient replication of rescued virus RNA may be due to restricted initiation as discussed in a previous section.

SYNERGIC EFFECTS AND BLOCKING OF ANTIVIRAL ACTION

SYNERGISM

Besides guanidine, 2-(α-hydroxybenzyl)benzimidazole (HBB) also inhibits the multiplication of picornaviruses selectively. Both compounds act on the synthesis of virus RNA. Results of experiments in which guanidine and HBB were added simultaneously to infected cells have established a synergic relationship between these selective inhibitors (Tamm and Eggers, 1962; Eggers and Tamm, 1963). These results are discussed in detail in the preceding chapter. Non-selective metabolic inhibitors have also been used to potentiate the action of guanidine. Such chemicals reduce both cell and virus biosynthesis in general and should be distinguished from synergism between two selective virus inhibitors. Potentiating effects on guanidine inhibition of poliovirus multiplication have been reported with ethionine (Ueda *et al.*, 1963a), a non-selective inhibitor of protein synthesis, and with 5-fluorouracil (Loddo *et al.*, 1963a), a non-selective inhibitor of nucleic acid synthesis. These are only two examples of any number of metabolic inhibitors which are non-selective and can potentiate the antiviral action of guanidine.

BLOCKING OF ANTIVIRAL ACTION

Antagonists capable of reversing the antiviral action of guanidine were first reported in 1964 (Dinter and Bengtsson, 1964; Lwoff and Lwoff, 1964b). The amino-acids which block the action of guanidine were identified when certain media were found to suppress guanidine inhibition

of foot-and-mouth disease virus multiplication (Dinter and Bengtsson, 1964) and poliovirus multiplication (Lwoff and Lwoff, 1964b). The effects of Eagle's minimum essential medium on inhibition of virus yield by guanidine are shown in Table 6. Similar results have been obtained with lactalbumin hydrolysate, which also reduces the inhibitory action of guanidine. These observations have led to an investigation of specific components of media with the discovery that several amino-acids, such as methionine, valine, leucine, and threonine, are effective in reducing the antiviral action of guanidine. Many other compounds containing methyl or ethyl groups are also active in reversing the effects of guanidine (Philipson *et al.*, 1966; Loddo *et al.*, 1966). Table 8 shows the relative antiguanidine activity of some of these compounds. The compounds in

TABLE 8. COMPOUNDS WHICH BLOCK THE ANTIVIRAL ACTION OF GUANIDINE

Compound	Concentration[a] 10^{-5} M	Virus yield % untreated control
1. Dimethylpropanolamine	5	85
2. Dimethylethanolamine	5	78
3. Methylethanolamine	5	23
4. Diethylethanolamine	5	22
5. Ethanolamine	5	18
6. Choline	5	3
Choline	50	72
7. Methionine	50	38
8. Norleucine	50	16
9. Isoleucine	50	2
10. Leucine	50	2
11. Valine	50	2

[a] Compounds were added simultaneously with 4×10^{-4} M guanidine to poliovirus type 1-infected HeLa cells in Eagle's minimum essential medium. Guanidine alone at this concentration reduced virus yield to 2% of untreated cultures.

From Philipson, Bengtsson and Dinter, 1966.

Table 8 are arranged in descending order of effectiveness in blocking virus inhibition by guanidine. In these experiments guanidine and the competitor were added simultaneously 30 min after inoculation. The most effective antagonists of guanidine action are the five derivatives of choline (compounds 1–5). As shown in Table 8, the first five compounds are more

effective at a concentration of 5×10^{-5} M than 10 times that concentration of any of the other compounds except choline and methionine. Of the normal constituents of Eagle's minimum essential medium (compounds 6–11) (Eagle, 1959), choline is the most effective antagonist.

Although the mechanism by which these compounds reverse guanidine action remains obscure, certain facts about their activity are known. A derivative of methionine and choline known to be an intermediate in transmethylation, S-adenosylmethionine, is ineffective in reversing inhibition of virus multiplication by guanidine (Philipson *et al.*, 1966). However, it is not clear that S-adenosylmethionine can enter the intact cell. Since methylguanidine is less active than guanidine, the possibility exists that guanidine is methylated in cells with the result that its antiviral action is partly destroyed. This mechanism of antagonism is, however, unlikely because guanidine is not methylated in the cell (Philipson *et al.*, 1966; Lwoff, 1965).

Philipson and co-workers (1966) have also reported synergism between guanidine antagonists. At a concentration of 10^{-4} M, dimethylethanolamine does not reverse the action of 10^{-3} M guanidine, whereas the simultaneous addition of 5×10^{-4} M norleucine and 10^{-4} M dimethylethanolamine causes 84% reversal of guanidine action. This enhancement of dimethylethanolamine reversion has been demonstrated with other amino-acids as well, but norleucine is the most active. Mosser *et al.* (1971) have suggested that several classes of guanidine antagonists exist and that synergism can be demonstrated between the different classes of anti-guanidines. Synergic effects were demonstrated between choline and methionine, but not between choline and dimethylethanolamine.

Dinter and Bengtsson (1964) have shown that lactalbumin hydrolysate does not suppress the virus-inhibitory activity of another selective inhibitor of picornavirus multiplication, 2-(α-hydroxybenzyl)benzimidazole (HBB). Loddo *et al.* (1966) have reported that trimethylamine, tetramethylammonium iodide, and methionine have very little effect on the action of HBB. The reversal of virus inhibition by these compounds appears to be limited to guanidine.

Choline at a concentration of 0.5 mM is capable of blocking nearly completely the moderate inhibitory effects of relatively low concentrations of guanidine such as 0.3–0.4 mM (Table 8). However, the marked inhibitory effects of 1.0–1.5 mM guanidine are affected only slightly or not at all by very high concentrations (40 mM) of choline (Mosser *et al.*, 1971). Philipson and co-workers (1966) have found that none of the antagonists support the growth of guanidine-dependent poliovirus in HeLa cells in

the absence of guanidine. However, Loddo *et al.* (1966) and Lwoff and Lwoff (1965) have reported that several guanidine antagonists support the multiplication of guanidine-dependent mutants of poliovirus in KB cells. This discrepancy may be due to the use of different cell types, since the effectiveness of the antagonism is profoundly influenced by the host cell (Philipson *et al.*, 1966). For example, the virus yield from guanidine-treated KB cells infected with poliovirus in the presence of 0.5 mM choline is 78% of control, but from monkey kidney cells it is less than 2% of control under the same conditions of treatment. Thus, choline blocks effectively the inhibitory action of guanidine in KB cells, but not in monkey kidney cells. Similarly, the amino-acids that are capable of blocking guanidine inhibition in KB cells do not have this effect in primary monkey kidney cells (Philipson *et al.*, 1966). Future work with guanidine antagonists may reveal a host cell specific process critical for virus multiplication. Mosser *et al.* (1971) have shown that the effectiveness of compounds in reversing the inhibitory action of guanidine in a given host cell is related to the sensitivity of poliovirus biosynthesis to inhibition by guanidine in that host cell. Poliovirus biosynthesis showed increasing sensitivity to inhibition by guanidine in different cells in the order: HeLa cells, LLC-MK_2 cells, and primary rhesus monkey kidney cells. More choline is required to produce an equivalent degree of blocking or reversal in cells in which the virus is more strongly inhibited by guanidine.

PROTECTIVE EFFECTS IN ANIMALS

Guanidine is ineffective in the chemotherapy of poliovirus infection of animals (Rightsel *et al.*, 1961; Barrera-Oro and Melnick, 1961; Melnick *et al.*, 1961; Dixon *et al.*, 1965). Non-toxic doses of either guanidine hydrochloride or the guanidine salt of hydroxyaminomethylene malononitrile do not decrease the incidence of disease in polio-infected monkeys (Rightsel *et al.*, 1961; Barrera-Oro and Melnick, 1961). The toxic dose of guanidine hydrochloride for monkeys is between 30 and 50 mg/kg/day, very similar to the toxic dose for humans (Cherington and Ryan, 1968). One of the reasons for the ineffectiveness of guanidine in chemotherapy may be the difficulty in maintaining an adequate blood level (Melnick *et al.*, 1961). Studies by Dixon and co-workers (1965) show that the half-life of the drug in rats is 2.1 hr and somewhat longer in dogs. The short half-life of guanidine in animals probably reflects rapid renal excretion, as demonstrated in the mouse (Rightsel *et al.*, 1961). Another factor which reduces the chemotherapeutic effectiveness of guanidine in animals is the rapid

development of drug-resistant mutants of poliovirus in animals (Barrera-Oro and Melnick, 1961; Melnick *et al.*, 1961).

Although attempts to immunize monkeys against paralytic poliomyelitis have been made with guanidine-dependent mutants of poliovirus (Loddo *et al.*, 1963c; Loddo *et al.*, 1964a; Loddo *et al.*, 1965), this approach has been superseded by successful immunization with live attenuated poliovirus.

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CHAPTER 4

PURINES AND PYRIMIDINES

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THE IMPORTANCE OF NUCLEIC ACIDS IN ANTIVIRAL CHEMOTHERAPY

THE discovery by Stanley (1935) that infectious tobacco mosaic virus could be obtained as a crystalline nucleoprotein suggested the vital role of nucleoproteins in at least some viruses. Hershey and Chase (1952) clearly demonstrated that only the DNA of the T2 bacteriophage particle entered the bacterial cell on infection, while the protein coat remained at the cell surface. These observations suggested that the bacteriophage DNA carried and transmitted all of the genetic information that directed virus reproduction. Fraenkel-Conrat (1956) and Fraenkel-Conrat and Singer (1957) reported that infectious tobacco mosaic virus nucleoprotein prepared by chemical reconstitution of RNA from one strain of virus with protein from another strain of virus yielded progeny (following infection of plants) that were biologically similar to the virus strain from which the parent RNA was derived. These findings strongly indicated that the nucleic acid in the virus nucleoprotein is the sole vital genetic determinant of the virus particle. A number of additional infectious crystalline nucleoproteins from other plant viruses have been isolated. Among the animal viruses, infectious crystalline nucleoproteins from poliomyelitis virus (RNA) (Schaffer and Schwerdt, 1955), encephalomyocarditis virus (RNA) (Faulkner *et al.*, 1960), Coxsackie virus (RNA) (Mattern and duBuy, 1956), and polyoma virus (DNA) (Murakami, 1963) have also been isolated. Although, in general, viruses contribute little to their own intracellular replication other than the genetic information which directs the host cell's metabolic machinery to the production of new viral components, some viruses do

contain, within the virion, enzymes which are probably required for the ultimate production of progeny virus. For example, vaccinia virus has been found to contain its own DNA-dependent RNA polymerase (Kates and McAuslan, 1967a, 1967b) which regulates the transcription of the RNA that serves as template for the synthesis of the uncoating protein and the early virus-specific enzymes which are prerequisite for viral DNA replication (Giorno and Kates, 1971). Vaccinia virus has also been shown to contain a nucleotide phosphohydrolase with affinities for a number of nucleotide triphosphates (Gold and Dales, 1968). Temin and Mizutani (1970) and Baltimore (1970) have observed that the virions of RNA tumor viruses contain an RNA-dependent DNA polymerase. These viruses also contain a double-stranded nucleic acid-dependent DNA polymerase (Spiegelman *et al.*, 1970a; Mizutani *et al.*, 1970; Spiegelman *et al.*, 1970b), DNA endonuclease activity (Mizutani *et al.*, 1970) and polynucleotide ligase and DNA exonuclease activity (Mizutani *et al.*, 1971). These enzymes give the virus all activities necessary to transform information from RNA to double-stranded DNA and to integrate it into the host cell DNA. A preliminary report by Burlingham *et al.* (1970) indicates that purified pentons from adenovirus type 2 also contain endonuclease activity, but DNA exonuclease activity has not been found either in the pentons or in the intact virions.

Extensive chemical and biological studies on viruses have clearly indicated that all examined to date may be characterized as follows:

1. All are obligate intracellular parasites which divert the host cell's metabolism to the production of viral nucleic acid and protein. The virus may contribute little to its own intracellular replication, except genetic control of the metabolism of the host cell, to achieve the parasite's (virus's) replication. In most naturally occurring virus infections in nature these activities proceed without lethal or even serious and grossly apparent damage to the host. Only when the virus multiplication takes place in vital and not easily replaceable cells does the well adapted virus parasite do serious and obvious harm (e.g., extensive infection and cell destruction in the gastrointestinal epithelium by poliomyelitis virus is usually clinically inapparent, but poliomyelitis virus infection of the motor neurones or other vital cells in the central nervous system often produces severe clinical disease and may be life threatening).

2. The one aspect common to and probably vital to all viruses is their nucleic acid, either RNA or DNA. All plant viruses have only RNA

while bacteriophages and animal viruses have either RNA or DNA. While nucleic acid type is a useful aid in virus classification, it may not be a fundamental distinguishing characteristic (e.g., both RNA and DNA oncogenic viruses of animals are known).

Since the common and obviously vital thread to the chemical structure of viruses is their nucleic acids, searches for specific inhibitors of functional replication of these nucleic acids as antiviral agents was clearly indicated to all investigators early in the search for antiviral drugs. With but few minor exceptions, DNAs are made up of adenine, guanine, cytosine and thymine (5-methyluracil) and RNAs are made up of adenine, guanine, cytosine and uracil. Therefore, specific antagonists of these purine and pyrimidine bases and their ribosides and ribotides are obvious potentially useful compounds to examine for antiviral activity.

A number of reviews on antiviral chemotherapy have appeared since 1955, some of which covered purine and pyrimidine analogs and antagonists in detail, and are of interest to the reader for additional historical background, points of view and approaches to the problem (Matthews and Smith, 1955; Staehelin, 1959; Todd, 1959; Prusoff, 1963; Skoda, 1963; Pienta and Groupé, 1964; Thompson, 1964; Elion and Hitchings, 1965; Kaufman, 1965a; Prusoff *et al.*, 1965; Welch, 1965; Prusoff, 1967; McFadzean, 1969; Goz and Prusoff, 1970; Osdene, 1970; and Schabel, 1970).

Larin (1967), in discussing the problems of searching for antiviral drugs, has correctly observed and stated that "The discovery and subsequent development of the first synthetic centrally active drugs, diuretics, sulfonamides, cardiac glucosides and antibiotics, such as penicillin, were in no way influenced by any fundamental knowledge of the processes with which they interfere." Few would seriously question the validity of Larin's statement. In fact, empirical search has been the keystone of both initial discovery and following improvement of nearly all useful chemotherapeutic agents. However, man, as a rational animal, continually seeks to improve his lot and solve his problems, including his medical problems, with logic, and orderly thinking and viral chemotherapy seem ripe for this logical approach via the purine and pyrimidine analogs and antagonists.

PURINES AND PYRIMIDINES AS ANTIVIRAL AGENTS

Since we are clearly in the age of "chemical biology" or "molecular biology", a consideration of virus chemotherapy organized on a chemical

basis seems indicated and we have considered the published reports on the antiviral activity of purines and pyrimidines on the basis of chemical structure as outlined in Table 1.

ANTIVIRAL ACTIVITY OF PURINES AND PYRIMIDINES IN VIRUS-INFECTED CELL CULTURE SYSTEMS

Probably the first report on inhibition of virus growth by a purine or pyrimidine was that of Thompson *et al.* (1949) who observed a small but reproducible inhibition of multiplication of vaccinia virus in chick embryo cell cultures by several 5-substituted uracils, e.g., 5-bromo- and 5-hydroxy-, and by 2,4-dithiothymine. At about the same time Nickell *et al.* (1950) reported that 8-azaguanine inhibited the growth of tissue culture of virus tumors of *Rumex acetosa* L. (sorrel) which carry the virus *Aureogenus magnivena* Black. This inhibition of tumor cell growth by 8-azaguanine could be reversed by guanine. Direct inhibition of virus growth or metabolism was not demonstrated, however. At the same time Matthews (1951) was studying the effect of a number of substituted purines on several plant viruses in a number of different intact plants. He observed inhibition of virus lesions following spray application or watering on the soil of 8-azaguanine in solution against lucerne mosaic virus or tobacco mosaic virus on *Nicotiana tabacum* var. White Burley and delay in appearance of systemic infection with cucumber mosaic virus in cucumbers, pea mosaic virus in peas, and tobacco mosaic virus in tomatoes.

In late 1950 Thompson *et al.* (1950) reported that 2,6-diaminopurine inhibited growth of vaccinia virus in chick embryo tissue cultures and this inhibitory activity was reversed by adenine, yeast nucleic acid, adenylic plus guanylic acids, coenzyme 1 and hypoxanthine, but not by xanthine or uracil.

Extensive and varied studies have been carried out on the antiviral properties of purines and pyrimidines against plant viruses. However, since our purpose in this chapter is to consider purines and pyrimidines as antiviral drugs against virus infections in man, no additional discussion of these compounds against plant viruses nor against animal viruses in non-mammalian cell cultures will be included. These early references to the activity of purines and pyrimidines against plant viruses or against animal viruses, in other than mammalian cell cultures, were included for historical interest.

The reported antiviral activities of purines and pyrimidines against animal and human viruses in mammalian cell culture systems are listed in

Tables 2–20. Several arbitrary decisions for inclusion in these tables are listed below:

1. We have attempted to limit these activity tabulations to reported studies in which the test compound was added to the cell cultures at the same time or after the addition of the infectious virus. This is consistent with our view that therapy follows infection and it was also done to avoid the difficulties of data interpretation imposed by drug damage to receptor sites and other often discussed but similarly vague and subjective considerations. No mention is made of any reports on the chemical destruction of extracellular virus.
2. Since this discussion is oriented to antiviral activity of purines and pyrimidines against viruses causing human illness, no reports of antiviral activity against bacteriophages, plant viruses or insect viruses are included in the tables or following discussion. We have further limited the reported cell culture antiviral studies to those done in mammalian cell cultures. For these reasons the reports listed in Tables 2–20 should not be considered to be a complete and total record of the published literature, but rather to be selected to contain information with the greatest likelihood of indicating possible antiviral activity of these compounds against virus infections in man.
3. Andrewes and Pereira (1967) and Wilner (1969) have been used as the reference sources for questions regarding viral taxonomy, nomenclature and classification according to characteristic type of nucleic acid (RNA or DNA virus).
4. The types of cells used in the cell culture studies listed in Tables 2–20 have been arbitrarily condensed. References to rodent cells contain those using cells from all common laboratory rodents such as mice, rats, guinea pigs, hamsters, and (by accepting outdated zoological classification) rabbits.

PURINES

The reported antiviral activity in mammalian cell culture of the 51 purines listed in Tables 2 through 10 may be summarized as follows:

Purines Tested Against:

DNA virus(es) only		RNA virus(es) only		Both DNA and RNA viruses			
				DNA		RNA	
Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
13	17	2	9	7	3	4	6

Of the 40 compounds tested against one or more DNA viruses, 20 (50%) were active while of the 21 tested against one or more RNA viruses, 6 (29%) were active.

PYRIMIDINES

The reported antiviral activity in mammalian cell culture of the 165 pyrimidines listed in Tables 11 through 20 may be summarized as follows:

Pyrimidines Tested Against:

DNA virus(es) only		RNA virus(es) only		Both DNA and RNA viruses			
				DNA		RNA	
Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
27	27	23	8	40	40	10	70

Of the 134 pyrimidines tested against one or more DNA viruses, 67 (50%) were active, while of the 111 tested against one or more RNA viruses, 33 (30%) were active.

We believe that no sweeping conclusions are indicated from these tabulations of reported data from antiviral testing in cell culture except to note that antiviral activity against a variety of human DNA and RNA viruses in mammalian cell culture systems has been repeatedly reported and its diversity in time, virus types, cell types, interlaboratory variation, and last but not least, chemical classes and structures in relation to current knowledge on the importance of purine and pyrimidine metabolism in viral reproduction clearly indicate the reality of chemical (drug) interference with natural processes in virus growth and the clear promise of practical antiviral chemotherapy against both RNA and DNA viruses.

It is of interest in this regard to point out that the thiosemicarbazones with unequivocally established activity against DNA viruses, especially the pox viruses, have been generally considered to be restricted in inhibitory activity to DNA viruses. However, Bauer *et al.* (1970) have reported that 1-methylindole-2,3-dione 3-thiosemicarbazone (Methisazone)

is active against a variety of both RNA and DNA viruses, and they and others (Schabel, 1965) have pointed out that experimental evidence is now present indicating the possibility of developing broad spectrum antiviral drugs.

All virus chemotherapists know that extensive searches for antiviral agents have been carried out throughout the world for about the past 20 years. Much of this work has been negative and has not been reported in the scientific literature. For these and other reasons, we recognize that the antiviral activity listed in Tables 2 through 20 is a selected body of data. Only a few studies included planned synthesis and testing based on likely structure–activity relationships among the compounds tested, but some studies of this kind have been conducted and many are indicated in light of recent progress and current knowledge.

The most interesting of the active purines included in Tables 2 through 10 is 9-β-D-arabinofuranosyladenine (ara-A). Since it received more extensive and internally controlled study than any of the other purines, it may not be unique among the more active purines but, at this point in time, it is the most promising candidate for further study leading to clinical trial in man (Schabel, 1968).

Much clearer progress has been achieved with the pyrimidines. IDU and ara-C are useful drugs in treating some DNA virus infections in man and their clinical use was preceded by demonstrated antiviral activity in cell culture.

The data presented in Tables 2 through 20 clearly document the current availability of established laboratory procedures using mammalian cells in culture for carrying out structure–activity studies on candidate antiviral drugs. Such studies are essential for the most logical and rapid progress in advancing drug control of virus infections in man.

ANTIVIRAL ACTIVITY OF PURINES AND PYRIMIDINES *IN VIVO* IN EXPERIMENTAL SYSTEMS

The reported activities of purines and pyrimidines *in vivo* in virus-infected laboratory animals are listed in Tables 21 through 39.

PURINES

The antiviral activity *in vivo* of the 40 purines studied and reported is listed in Tables 21 through 29 and may be summarized as follows:

Purines Tested for *In Vivo* Antiviral Activity Against:

DNA virus(es) only		RNA virus(es) only		Both DNA and RNA viruses			
				DNA		RNA	
Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
3	1	0	9	2	25	11	16

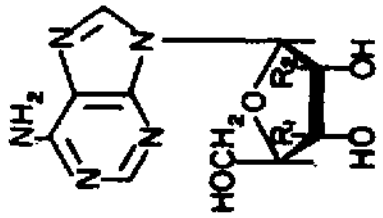
Of the 31 purines tested against DNA viruses, 5 (16%) were active and of the 36 purines tested against RNA viruses, 11 (31%) were active.

Among the natural purines, only adenine has been tested and only against Semliki Forest virus in mice. It was reported to be inactive (Table 21).

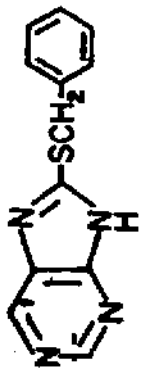
Among four 6-aminopurines substituted at the 2-carbon which have been tested for *in vivo* activity against a limited spectrum of RNA and DNA viruses only one unconfirmed report of the antiviral activity of 2-aminoadenine (**1**) against Russian spring–summer encephalitis was found (Table 22). Three of the compounds were tested for *in vivo* activity against vaccinia virus in mice and all were inactive.

Two 6-aminopurines substituted at the amino group have been studied *in vivo* against a limited spectrum of viruses. It is of interest that 6-(2,2-dimethylhydrazino)purine (**2**) significantly inhibited virus-induced splenomegaly and also reduced the virus content in the spleens of mice treated with drug 24 hr after inoculation of Friend leukemia virus (FLV), an RNA virus, but it was inactive *in vivo* against vaccinia virus, a DNA virus (Table 23).

A few adenine nucleosides have been tested for *in vivo* antiviral activity in experimental systems (Table 24) and activity against several DNA viruses has been reported. Walton *et al.* (1969) found both 2'- and 3'-*C*-methyladenosine (**3** and **4**) to be active against vaccinia in mice. Privat de Garilhe and De Rudder (1966a) (Table 5) first observed antiviral activity of 9-β-D-arabinofuranosyladenine (ara-A, **5**) *in vitro* and this activity was confirmed and extended by these workers and others (Table 5). The compound has been shown to be active *in vivo* against herpes simplex and vaccinia viruses inoculated intracerebrally (i.c.) when treatment is by the



3: R_1 = H, R_2 = CH_3
4: R_1 = CH_3, R_2 = H



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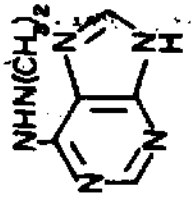

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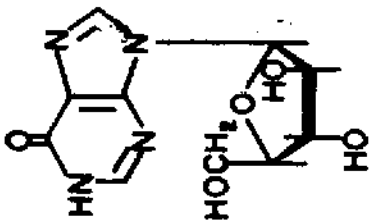

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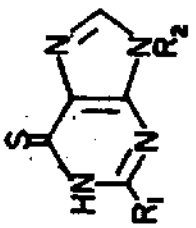

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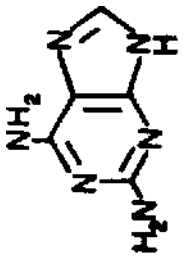

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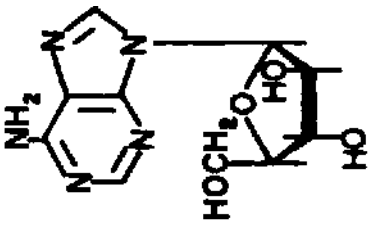

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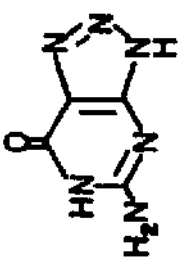

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intraperitoneal (i.p.), oral (p.o.) or percutaneous (p.c.) route. It is active against experimental herpes keratitis when treatment is either topical or parenteral. The antiviral activity against experimental infections of ara-A and its indicated promise as a useful antiviral drug have been reviewed by Schabel (1968). To our knowledge ara-A has not been studied against any virus infections in man to this time (1971).

A number of other variously substituted purine analogs have been tested for *in vivo* activity against a variety of DNA and RNA viruses (Tables 25–29). 9-β-D-Arabinofuranosylhypoxanthine (**6**), the deamination product of ara-A, was reported active against i.c. inoculated herpes simplex in mice (Table 26). 8-Azaguanine (**7**) was reported to be active against i.c. inoculated lymphocytic choriomeningitis virus (RNA) in mice (Table 28). A number of variously substituted 6-thiopurines (**8**) have been reported to show antiviral activity *in vivo* against FLV (Table 27). Among eight additional miscellaneous purines tested for *in vivo* antiviral activity against a number of DNA and RNA viruses, only 8-(benzylthio)purine (**9**) was reported to be active against vaccinia virus (Table 29).

PYRIMIDINES

Very extensive *in vivo* studies on the antiviral activity of pyrimidines have been carried out in recent years, beginning in the mid 1940's and increasing progressively as positive results became more numerous and the probability of useful application of these laboratory successes to human medicine become more obvious.

Published reports on the observed *in vivo* antiviral activity of these pyrimidines are listed in Tables 30–39. The antiviral activity *in vivo* of the 115 pyrimidines studied and reported is listed in Tables 30 through 39 and may be summarized as follows:

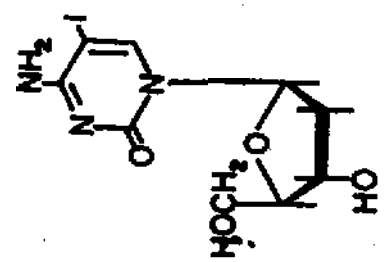
Pyrimidines Tested for *In Vivo* Antiviral Activity Against:

DNA virus(es) only		RNA virus(es) only		Both DNA and RNA viruses			
				DNA		RNA	
Active	Inactive	Active	Inactive	Active	Inactive	Active	Inactive
49	27	9	10	12	8	8	12

Of the 96 pyrimidines tested against one or more DNA viruses *in vivo*, 61 (64%) were active while of the 39 pyrimidines tested *in vivo* against one or more RNA viruses 17 (44%) were active.

A large number of 5-halo substituted and 5-alkyl substituted uracils and their nucleotides, and variously substituted analogs of cytosine have antiviral activity *in vivo*, particularly against vaccinia (DNA) and herpes simplex (DNA) viruses. 5-Fluorouracil (**10**) was active against lymphocytic choriomeningitis, an RNA virus, in mice, but its 2′-deoxyribonucleoside (**15**) was inactive against both DNA and RNA viruses. Both the 2′-deoxyribonucleoside (**16**) and the arabinonucleoside (**24**) of 5-chlorouracil are active against herpes simplex when applied topically to the virus-infected cornea of the rabbit (Table 31). 5-Bromouracil (**11**) and 5-iodouracil (**12**) are inactive, presumably because they are not anabolized to their nucleotides, but their arabinonucleosides (**25** and **26**) and their 2′-deoxyribonucleosides (**17** and **18**), which are known to be phosphorylated *in vivo*, are active against herpes simplex when applied topically. 2′-Deoxy-5-iodouridine (**18**, IDU), which is discussed in more detail later, is active against a number of DNA viruses and at least one RNA virus. The effect of the substituent at C-5 of the uracil nucleosides thus determines their activity. 5-Fluorouracil (**10**) and its nucleosides which sterically resemble uridine or 2′-deoxyuridine, since the van der Waals radius of the fluoro group is close to that of hydrogen, do not inhibit DNA viruses. The larger halogens (Cl, Br, I) at C-5, whose van der Waals radii approximate that of the 5-methyl group of thymidine, impart activity against DNA viruses to the uracil nucleosides containing them. The triacetate of 1-β-D-arabinofuranosyl-5-chlorouracil (**24**) and the diacetates of IDU and 2′-deoxy-5-iodocytidine probably owe their activity to *in vivo* deacetylation by non-specific esterases. 2′-Deoxy-5-iodocytidine (**28**) itself, which is more soluble and more stable than IDU, is deaminated *in vivo* to IDU. The lack of activity of 5-iodouridine shows the importance of the sugar moiety to activity, again emphasizing the relationship to the 2′-deoxyribonucleoside thymidine of DNA.

5-Trifluoromethyl- (**20**), 5-ethyl- (**21**) and 5-propyl-2-deoxyuridine (**22**), and 1-β-D-arabinofuranosylthymine (**27**), all of which also resemble thymidine, were active topically against herpes simplex virus (Table 32). The uracil mustard (**13**) was active against vaccinia (DNA) and influenza A (RNA) but not against herpes simplex (Table 33). 5-Amino- (**33**) and 5-hydroxy-6-methyluracils (**34**) were both active against vaccinia, but other 5-amino-6-substituted uracils were not. 5-Formylaminouracil (**14**) was also active against vaccinia, and 2′-deoxy-5-methylaminouridine (**23**)



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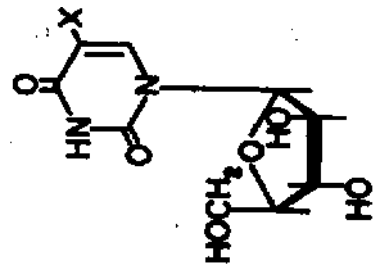
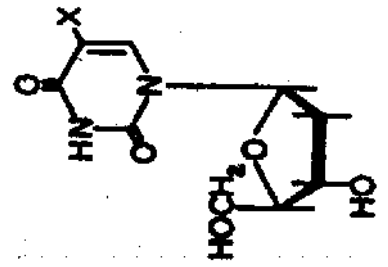
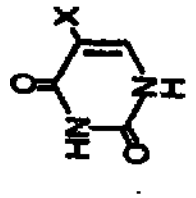


24: X = Cl
25: X = Br
26: X = I
27: X = CH_3



15: X = F
16: X = Cl
17: X = Br
18: X = I (IDU)
19: X = CH_3
20: X = CF_3
21: X = CH_2CH_3
22: X = $(CH_2)_2CH_3$
23: X = $NHCH_3$



10: X = F
11: X = Br
12: X = I
13: X = $NCH_2CH_2Cl_2$
14: X = NHCHO

(36)

(35)

(33: X = NH_2; 34: X = OH)

(29: X = H (ara-C); 30: X = Cl; 31: X = Br; 32: X = I)

was active topically against herpes simplex. Among the N-substituted uracil derivatives, 3-mercaptomethyluracil (**35**) was active against vaccinia and influenza A, and 1-allyl-3,5-diethyl-6-chlorouracil (**36**) was active against herpes simplex (Table 34). In contrast to the adenosines, 3′-*C*-methylcytidine (**37**) was active against vaccinia, but the 2′-*C*-methyl isomer (**38**) was not (Table 35). 1-β-D-Arabinofuranosylcytosine (ara-C, **29**), which is highly active against a number of DNA viruses, but inactive against RNA viruses, will be discussed in more detail. A number of phosphate esters of ara-C are also active (Table 35), but their activity is probably due to *in vivo* conversion back to ara-C itself. Some of the halogenated derivatives of ara-C (**30–32**) and 2′-deoxycytidine (**28**) are also active against DNA viruses. Methylation at N-3 of ara-C (to give **39**) does not inactivate it.

Thompson *et al.* (1951) reported that 5-(2,4-dichlorophenoxy)-2-thiouracil (**40**) was the most effective of a series of 5-aryloxy-2-thiouracils in protecting mice against vaccinia inoculated i.c. or intranasally (i.n.). A number of these 2-thiouracils were also active against Semliki Forest (an RNA virus) in mice (Table 36). Several 2-amino-5-phenoxy-4-thiopyrimidines (**41**) were also found active against vaccinia (Table 39).

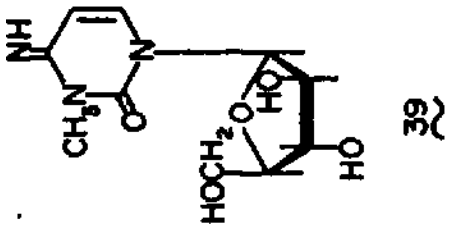
6-Azauracil (**42**) and its ribonucleoside (**43**) were both active against vaccinia, and 6-azauracil (42) was active against influenza A, an RNA virus. 6-Azacytidine was inactive against both viruses (Table 38).

A series of 5-alkyl derivatives of barbituric acids (**44**) were found inactive against St. Louis encephalitis and polio, both RNA viruses, but a related series of compounds were active against Japanese encephalitis (Table 39). A 2-thiobarbituric acid (**45**) was active against influenza A1, A2, and B and parainfluenza 1, but inactive against influenza A (PR8) (Table 39).

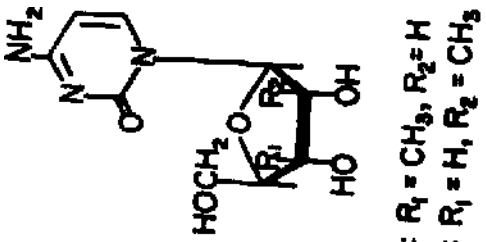
THE BIOCHEMICAL BASIS OF THE ANTIVIRAL ACTIVITY OF PURINES AND PYRIMIDINES

Since a number of excellent reviews have considered in detail the problems of defining the mechanism of antiviral agents, including the pyrimidines and purines (Staehelin, 1959; Elion and Hitchings, 1965; Kaufman, 1965a; Prusoff, 1967; Goz and Prusoff, 1970), the current status of the art will be reviewed only briefly here.

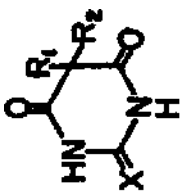
An antiviral agent could act by extracellular inactivation of the virus, by inhibition of absorption or penetration of cells by the virus, or by inhibition of an intracellular event. Although little is known about the mechanism of action of many of the pyrimidines and purines that have



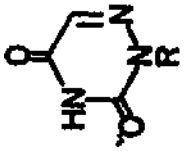
39



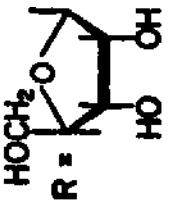
37: $R_1 = CH_3$, $R_2 = H$
38: $R_1 = H$, $R_2 = CH_3$



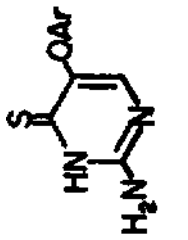
44: X = O
45: X = S



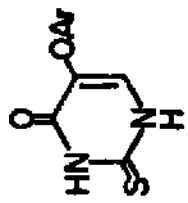
42: R = H

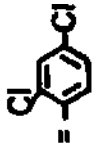


43: R =



41





40: Ar =

shown antiviral activity, 2'-deoxy-5-iodouridine (IDU) has been extensively investigated and found to inhibit a number of enzymes involved in DNA synthesis—thymidine kinase, thymidylate kinase, nucleoside diphosphate reductase, and DNA polymerase—and to be incorporated into DNA (Goz and Prusoff, 1970). Kaufman (1965a) has suggested that the action of IDU may be due to its preferential inhibition of specific virus-induced enzymes, but Goz and Prusoff (1970), after careful consideration of the many studies on IDU by Prusoff and coworkers and others, concluded that the primary site of inhibition of IDU is, in any event, subsequent to its incorporation into viral DNA in place of the thymidine moiety. On the other hand, the other pyrimidine that has shown good activity in man, 1-β-D-arabinofuranosylcytosine (ara-C), is poorly, if at all, incorporated into DNA (Chu and Fischer, 1965; Furth and Cohen, 1967; Momparler, 1969), but inhibits both nucleoside diphosphate reductase (Chu and Fischer, 1962; Evans *et al.*, 1964; Kim and Eidinoff, 1965) and DNA polymerase (Furth and Cohen, 1967; Momparler, 1969). It appears that the biologic activity of ara-C and its purine counterpart, 9-β-D-arabinofuranosyladenine—the most effective antiviral agent among the purines (Schabel, 1970)—is probably due to the inhibition of the latter enzyme (Furth and Cohen, 1967). Strong evidence for support of this contention is found in studies that show that cell lines resistant to other known nucleoside diphosphate reductase inhibitors (e.g., hydroxyurea, guanazole, and the thiosemicarbazones) are sensitive to ara-C and *vice versa* (Schabel *et al.*, 1971). However, it should be noted that the nucleoside diphosphate reductase of cells infected with pseudorabies virus is not as sensitive to regulatory controls as that of uninfected cells and this altered enzyme plus a decrease in deoxycytidine kinase activity are held to be responsible for the relative resistance of the infected cells to ara-C (Kaplan and Ben-Porat, 1970).

Regardless of the exact site of action of these agents, they specifically interfere with DNA synthesis of function, and they are primarily effective against DNA but not RNA viruses. Furthermore, it has been convincingly demonstrated in cell culture and in animals that they are effective at levels far below their toxic levels (Prusoff, 1967), indicating true specificity for viruses. 2-Thiouridine, which is active against RNA viruses, is thought to be incorporated into viral RNA rendering it biologically inactive (Slechta and Hunter, 1970). The 6-thiopurines, which have shown some antiviral activity, interfere with both RNA and DNA synthesis (Montgomery, 1970) and, perhaps as a consequence, are probably too toxic to be seriously considered for use in man.

THERAPEUTIC ACTIVITY OF PURINES AND PYRIMIDINES ON VIRUS INFECTIONS IN MAN

PURINES

Only one report has been made of a clinical trial for antiviral activity in man with a purine. Hall *et al.* (1968) reported treating eight patients ill with Dawson's inclusion body encephalitis (DIBE) (in which measles virus may have an etiological association) with 8-azaguanine at nontoxic doses via the intraventricular route. No clear evidence of clinical improvement in these 8-azaguanine-treated patients was seen, although a clinical impression of stabilization of the disease was obtained.

PYRIMIDINES AGAINST RNA VIRUS INFECTIONS IN MAN

The clinical reports on the use of pyrimidines in treating virus infections in man are listed in Table 40. With three exceptions, all of these therapeutic trials were against DNA virus infections. It is of great interest, however, to note that three reports from two different groups of investigators on the favorable therapeutic effect of 5-bromo-2′-deoxyuridine against subacute sclerosing panencephalitis (SSPE) or Dawson's inclusion body encephalitis (DIBE), both of which may be associated with measles virus either in a direct causal or some lesser but still closely involved manner. Specific drug treatment of SSPE and/or DIBE in relation to useful antiviral chemotherapy should be aggressively pursued and observed with critical judgment and great interest.

PYRIMIDINES AGAINST DNA VIRUS INFECTIONS IN MAN

WARTS—VERRUCAE

Hursthouse (1970) reported that daily use of a 5% 5-fluorouracil topical ointment, in an uncontrolled study, resulted in a complete disappearance of warts (previously uncontrolled by other methods) in twelve patients in 2–6 weeks. In three patients partial response was observed, in one no response was noted, and in two patients transient response occurred.

CYTOMEGALOVIRUS INFECTIONS

5-Fluoro-2′-deoxyuridine (FUDR) was reported by Cangir *et al.* (1967) to be effective in all of five critically ill children infected with clinically

(but not laboratory) established cytomegalovirus pneumonitis during anticancer drug-induced clinical remission of acute leukemia.

IDU was reported by Conchie *et al.* (1968) to have reduced the cytomegalovirus excretion in the urine and probable clinical improvement in a 14-week-old infant with congenital cytomegalovirus infection (laboratory established).

VARIOLA

Jaffari and Hussain (1969) observed 140 unvaccinated smallpox patients for antiviral activity of 6-azauridine. Patients were randomized into drug-treated and placebo-treated groups. They reported a 50% reduction in mortality in drug-treated patients with ordinary smallpox over placebo-treated controls and increases in life span of drug-treated patients dying of hemorrhagic, flat, or ordinary smallpox over placebo-treated control patients.

VACCINIA

IDU has been reported to have prophylactic antiviral activity in man against vaccinia virus. Calabresi *et al.* (1962) and Calabresi (1963, 1965) have reported that treatment of patients with IDU prevented primary takes following smallpox vaccinations in subsequently demonstrated susceptible individuals.

Two cases of vaccinia gangrenosa treated with IDU and Marboran have been reported (Calabresi, 1965, and Boughton *et al.*, 1969). In neither case was IDU judged to be usefully effective. Calabresi (1965) reported a third case of vaccinia gangrenosa treated with IDU and vaccinia immune globulin in which prompt and favorable clinical response followed treatment with IDU. This case was complicated, however, by intensive treatment, prior to the IDU therapy, with 6-azauridine, pyrazolo[3,4-*d*]-pyrimidin-4(5H)-one (Allopurinol) and colchicine. No clear indication of marked therapeutic activity of pyrimidines against generalized vaccinial infections in man is evident from reports of clinical studies to this time.

Two reports of useful therapeutic activity of IDU in treating vaccinial infection of the cornea have appeared (Vitiello, 1967; Jelinek, 1969).

VARICELLA-ZOSTER

The two viruses varicella (chickenpox) and zoster (shingles) are clearly related, if not identical, based on taxonomic, immunologic, epidemiologic

and other considerations. However, for purposes of this discussion we will consider chickenpox and shingles as separate clinical entities.

Treatment of Varicella (Chickenpox) with Ara-C. Calabresi (1965) first reported on favorable therapeutic response of a case of varicella virus infection to treatment with ara-C. A varicella eruption appeared in an infant about 6 weeks after amputation of an arm for an embryonal rhabdomyosarcoma in the hand. Intravenous treatment with ara-C was followed by clinical improvement, possibly but not clearly due to treatment with ara-C, since the course of the disease was improving when ara-C treatment was started.

Hall *et al.* (1968, 1969) reported that six of seven patients with progressive, severe generalized varicella infections (2 moribund) showed prompt clinical response to intravenous treatment with ara-C.

Prager *et al.* (1971) reported on a 6-year-old leukemic child under intensive antileukemic drug therapy who developed generalized varicella. Treatment with ara-C by the intravenous route resulted in prompt arrest of the progress of the disease followed by rapid recovery and healing of the lesions.

Zoster (Shingles). IDU. IDU has been used for topical treatment of zoster infections, and opinions vary about its therapeutic effect. McCallum *et al.* (1964) tested 52 patients suffering from zoster in a double-blind trial designed to show whether topical treatment with 0.1% IDU was a useful therapeutic measure. They concluded that there was no clear indication of therapeutic effectiveness.

Juel-Jensen (1970b) used 40% IDU in DMSO as a topical treatment of about 20 patients with herpes zoster infections and observed marked relief of pain and earlier healing of the lesions in IDU-treated patients, which was especially apparent in those 60 years of age or older.

Waltuch and Sachs (1968) reported that treatment of a patient with Hodgkin's disease who developed a generalized zoster infection responded promptly to treatment (presumably parenteral) with IDU. The clinical disease cleared rapidly and the cutaneous lesions healed promptly.

Ara-C. Wiernick and Serpick (1969) reported the development of a case of zoster infection in a patient with acute myelomonocytic leukemia shortly after an intensive course of ara-C as anticancer drug therapy. Seligman and Rosner (1970) reported a similar case in which generalized varicella developed in a 4-year-old leukemic child under maintenance

therapy with 6-thioguanine plus ara-C. If ara-C has activity against zoster, abortion of these cases should have resulted.

Kwaan *et al.* (1969) reported the development of a severe generalized zoster infection in a 65-year-old man under intensive drug treatment for a lymphoproliferative disorder. Treatment of this patient with ara-C by the intravenous route resulted in prompt arrest of the progress of the disease and quick healing of the lesions.

Chow *et al.* (1970) reported that intravenous treatment with ara-C of two cases of disseminated zoster complicating acute myelocytic leukemia and chronic lymphocytic leukemia resulted in excellent clinical response within 24 hr of start of treatment and clinical recovery from the infection after 6–7 days of continuous therapy. In addition, two cases of shingles without evidence of dissemination were abruptly aborted by intravenous infusions of ara-C for 3 days.

Other pyrimidines. Murthy and Testa (1966) reported topical treatment of zoster lesions with a 0.35% aqueous solution of 5-nitrouracil to be effective.

Myska *et al.* (1967) reported that oral treatment with 6-azauridine cured zoster keratitis and iridocyclitis in a single patient after 2 weeks of treatment. The etiology of the ocular lesions was presumed, based on a clinically apparent zoster infection shortly before the appearance of the ocular lesions.

HERPES (SIMPLEX)

Even a casual reading of Table 40 quickly reveals that the vast bulk of clinical testing of pyrimidines for antiviral activity in man has been involved with herpes infections. This is due to many things, among them (1) early observed and widely confirmed activity of IDU against herpes infections both *in vitro* and *in vivo* in experimental systems, and (2) frequent infections in man with degrees of severity ranging from mild to severe discomfort (cutaneous lesions) to severe infections threatening the sense of sight (herpes keratitis) to severe and life threatening (herpes encephalitis). Extensive chemotherapy trials in herpes keratitis with candidate antiviral agents probably were dependent upon the availability of useful drugs (especially IDU) and aggressive investigators committed to improving the *status quo* of traditional therapeutic measures.

IDU. Table 40 lists 22 favorable clinical reports of the effectiveness of topical IDU alone in treatment of herpes keratitis and only 2 negative

reports. Of 23 reports on the topical treatment of cutaneous, conjunctival, oral or genital herpes infections, 17 (74%) report favorable clinical response. Ninety-five percent (18 of 19) of clinical reports on the activity of parenteral IDU in treating herpes encephalitis are favorable.

Lest the reader accept, from the above tabulation of reports and discussion, that IDU is generally accepted by all observers to be widely and unequivocally effective against herpes keratitis, cutaneous herpes or herpes encephalitis, a word of caution is necessary. Favorable reports, especially clinical reports, may be made by enthusiasts or wishful thinkers. However, the results from numerous double blind studies carried out against herpes keratitis in man treated with IDU indicate its significant therapeutic activity and the burden of proof now rests on those who disclaim its effectiveness.

The exact truth regarding the effectiveness of IDU in treating cutaneous herpes is still not clear but if IDU is effective in herpes keratitis, as it surely is, then all that appears necessary to establish unequivocal effectiveness against cutaneous herpes is the collection of a greater body of experience and evidence.

Whether the effectiveness of IDU in treating herpes encephalitis is clearly established is still sharply questioned by some whose opinions claim earned respect. For example, Tomlinson and MacCallum (1970) state ". . . there is, so far, no convincing evidence that IUdR (IDU) influences the course of clinical herpes encephalitis". Despite this, we have included their report in Table 40 as one of the 14 reporting favorable clinical response in humans with herpes encephalitis treated with IDU. Most of the favorable reports were on single cases, although one (Rappel and Brihaye, 1969) contained 11 cases, 9 of which responded favorably to IDU treatment. Perhaps we may only conclude at this time that IDU appears to have sufficient promise for treatment of herpes encephalitis to indicate carefully controlled further trials. These should not be too long in coming since severe herpetic infections, including encephalitis, frequently accompany vigorous immunosuppressive anticancer drug therapy and immunosuppressive drug support to organ and tissue transplant patients, and are also seen in patients with naturally occurring disease of the lymphoreticular system.

If one considers the wide variety of types of clinical disease and to this adds the variation in therapeutic application of the drug, this clinical record of effective chemotherapeutic response of a number of herpes infections to IDU is indeed impressive and convincing.

Ara-C. Ara-C has been reported to be effective in treating herpes keratitis in man (Kaufman and Maloney, 1963b), herpes encephalitis (Chow *et al.*, 1970), and disseminated lesions, including some of parenchymal organs (Juel-Jensen, 1970c). The promise of ara-C as a useful drug against herpes virus infections is great, but its unequivocal practical utility must await further clinical evaluation.

Other pyrimidines. 5-Chloro-2′-deoxyuridine, 5-ethyl-2′-deoxyuridine, 1-allyl-3,5-diethyl-6-chlorouracil, and 6-azauridine have also been reported to be effective by topical or oral (6-azauridine) treatment in herpes keratitis in man (Table 40).

NATURALLY OCCURRING AND SELECTED RESISTANCE OF HERPETIC INFECTIONS TO IDU AND ARA-C

Kaufman and Maloney (1963b) reported that a patient with herpes keratitis resistant to treatment with IDU had a favorable response following treatment with ara-C. Laibson *et al.* (1963) isolated a strain of herpes virus from a human case of herpes keratitis which failed to respond to treatment with IDU. When this virus was used to induce experimental herpes keratitis in rabbits, it failed to respond to treatment with IDU.

Buthala (1964) and Underwood *et al.* (1964) reported that an IDU-resistant line of herpes virus was obtained on serial passage of the virus in cell culture in the presence of IDU. The IDU-resistant virus was sensitive to ara-C in cell culture. Similar serial passage of the IDU-sensitive parent virus in the presence of ara-C failed to yield a line of virus resistant to ara-C. Underwood *et al.* (1965) isolated herpes virus from the eyes of rabbits with experimental herpes keratitis treated with IDU or ara-C. The virus isolated from IDU-treated eyes was sensitive to ara-C but resistant to IDU in cell culture, while the virus isolated from ara-C-treated eyes remained sensitive to both ara-C and IDU in cell culture. Kobayashi and Nakamura (1964) isolated a line of herpes virus selected for resistance to IDU by serial passage of a line of virus (isolated from a human case of herpes keratitis) in rabbit kidney cell culture in the presence of IDU. When experimental keratitis was induced in rabbits with this IDU-resistant line of virus, it failed to respond to therapy with IDU. Kaufman and Heidelberger (1964) reported that 5-trifluoromethyl-2′-deoxyuridine (F_3TDR) is effective in treating experimental herpes keratitis in rabbits induced with an IDU-resistant strain of virus.

From the above observations, it is apparent that resistance of herpes

virus to IDU may occur in man without prior treatment with IDU, and certainly IDU-resistant lines of virus appear quickly under chronic exposure in the laboratory. IDU-resistant lines of virus are generally sensitive to ara-C and at least are as sensitive to F_3TDR as the parent IDU-sensitive line.

While no viruses selected for resistance to ara-C during chronic exposure to ara-C have been reported, they may be expected to appear, since the appearance of resistance to ara-C in tumor cells under chronic treatment is easily demonstrated (Wodinsky and Kensler, 1964; Dixon and Adamson, 1965).

HUMAN PHARMACOLOGY AND THERAPEUTICS

IDU and ara-C are the only compounds among the purines and pyrimidines discussed above that have had sufficient study as antiviral agents in man to warrant discussion here.

IDU

HERPES KERATITIS

The most commonly used form is a 0.1% solution dropped onto the infected eye once each hour during the day and once each two hours during the night. An ophthalmic ointment containing 0.5% IDU has been less frequently used.

GENERALIZED VARICELLA, ZOSTER AND HERPES ENCEPHALITIS

Treatment of DNA viral infections in man with systemic IDU has been generally limited to patients with altered immune defenses or with fulminant infections that regularly have a high mortality rate. Generalized zoster or varicella complicating neoplastic disease and spontaneous herpes encephalitis fall into each of these categories.

Dosage, Schedule and Route of Administration. IDU has been administered in doses varying from 14 to 600 mg/kg per course of therapy with a course lasting 2½ to 19 days (Breeden *et al.*, 1966; Buckley and MacCallum, 1967; Evans *et al.*, 1967; Marshall, 1967; Bellanti *et al.*, 1968; Partridge and Millis, 1968; Golden *et al.*, 1969; Charnock and Cramblett,

1970; Meyer *et al.*, 1970; Nolan *et al.*, 1969; Wenzl and Rubio, 1970; Overgaard *et al.*, 1971). It has been given as a lumbar intrathecal injection, unilateral arterial perfusion and intravenous infusion.

Most adults or children can tolerate 80–100 mg/kg/day for 5 days. Because of its rapid metabolic degradation to 5-iodouracil, the IDU should be given as a continuous intravenous infusion (Calabresi *et al.* 1961).

Unilateral arterial perfusion is not recommended except in rare circumstances when an isolated lesion is to be treated. Intrathecal injection for the treatment of herpes encephalitis appears to have little rational basis since (a) the infection is both deep and superficial and the drug probably does not penetrate more than a few millimeters and (b) unless the drug is injected in a volume at least equal to 10% of the estimated CSF volume, it is unlikely that an effective concentration of drug will reach from the lumbar subarachnoid space past the level of the basal cisterns (Reiselbach *et al.*, 1962). This latter problem might be avoided by the insertion of bilateral intraventricular catheters.

It appears that systemic IDU may have a beneficial effect in herpes encephalitis when given as long as two weeks after the onset of symptoms. This effect can be seen in both improved survival rates and diminished residual neurological deficit in the survivors.

TOXICITY

The spectrum of IDU toxicity as reported by Calabresi *et al.* (1961) is bone marrow depression, stomatitis and alopecia. Transient hepatic enzyme elevations have also been seen (Bellanti *et al.*, 1968). The toxicity is related to the total dose administered (per 5-day course) and the schedule of administration. In the recommended dose of 80–100 kg/mg/day for 5 days as a continuous infusion, most patients will have moderate and a few will have marked myelotoxicity. Since the bone marrow suppression lasts only one or two weeks, vigorous support with platelet transfusions and antibiotics, if indicated, should prevent bleeding and secondary bacterial infection. If myelosuppression is present prior to the start of IDU therapy, it is likely to be more severe and prolonged and dose and duration should be reduced. IDU should also be given cautiously if other myelosuppressive drugs are being given concurrently.

Since IDU has carcinogenic and teratogenic potential, careful consideration must be given before it is administered to pregnant women or to patients whose disease can be expected to be self-limited.

ARA-C

Ara-C has been used to treat localized and disseminated zoster (Wiernik and Serpick, 1969; Chow *et al.*, 1970), disseminated and fulminating varicella (Calabresi, 1965; Hall *et al.*, 1968; Kwaan *et al.*, 1969; Prager *et al.*, 1971), herpes keratitis (Kaufman and Maloney, 1963b), disseminated herpes (Juel-Jensen, 1970c, 1970d) and herpes encephalitis (Chow *et al.*, 1970).

DOSAGE, SCHEDULE AND ROUTE OF ADMINISTRATION

The optimum dose of ara-C for the treatment of viral infections is not known. In theory, a dose that would provide viral stasis without immunosuppression would be ideal. Daily doses from 10 to 120 mg/M^2/day for one to 7 days have been reported. A reasonable starting dose for severe infections such as disseminated varicella or herpes encephalitis is an intravenous dose of 40 mg/M^2, continued for 4 to 5 days. If no response is seen after 48 hr, the dose may be doubled.

Initial experience with ara-C in the treatment of varicella and zoster has been encouraging, as measured by the arrest of formation of new cutaneous lesions and the early subsidence of pain. Insufficient patients with herpes keratitis, disseminated herpes or herpes encephalitis have been treated to make a judgment of the benefit of ara-C in these diseases.

TOXICITY

The toxicity of ara-C is dependent upon total dose, schedule and duration of administration. At the recommended dose of an intravenous loading dose of 40 mg/M^2 followed by 40 mg/M^2/day as a 4- to 5-day infusion, moderate hematopoietic toxicity can be expected. Higher doses regularly produce severe though temporary myelosuppression, while doses of 10–20 mg/M^2/day for 2–3 days can be expected to produce little or no toxicity. Nausea and vomiting may be seen at the higher doses. Hepatic enzyme and bilirubin elevations occasionally may also be seen.

Previous or concurrent administration of other myelosuppressive drugs may increase the sensitivity of the marrow to ara-C, and drug dosage and duration should be decreased. As with IDU, careful consideration of possible oncogenic and teratogenic potential must be taken before ara-C is given to pregnant women or patients with normal life expectancy and mild viral diseases.

DISCUSSION AND GENERAL CONCLUSIONS

From the analysis of the extensive experimental and clinical data presented, several facts are clearly apparent.

A. Experimental methods are currently available which will detect antiviral activity of purines and pyrimidines (as well as other antiviral agents) in both *in vitro* mammalian cell culture virus-host systems and *in vivo* in experimental animals infected with human viruses adapted to and pathogenic for selected laboratory animals.

B. These experimental systems have selected candidate purines and pyrimidines (and other agents) which have ultimately been shown to possess useful therapeutic activity against a few DNA virus infections in man.

C. Available laboratory methods are sufficiently reliable, quantitative and reproducible to allow meaningful chemical structure–activity relationship studies between congeners of compounds with demonstrated antiviral activity. This is the keystone of logical progress in this important and obviously vulnerable but still uncontrolled area in human medicine.

D. The number of purines and pyrimidines with demonstrated activity against RNA viruses *in vitro* suggests that RNA viruses may also be vulnerable to aggressive attempts to develop effective therapeutically useful antiviral agents against them. Obviously the RNA virus infections of man represent the major part of the human virus disease problem most in need of chemical prophylactic and/or therapeutic control, since easily practical immunologic prophylaxis does not appear likely to be attainable. The present status of a number of purines and pyrimidines with demonstrated *in vitro* activity against several RNA viruses is not appreciably different from the status of IDU when Herrmann (1961) first reported its *in vitro* activity against herpes and vaccinia in cell culture. The *in vitro* and *in vivo* antiviral activity of a variety of purines and pyrimidines against a number of RNA and DNA viruses clearly shows that further work with these and related compounds is indicated and such effort promises further success in the development of effective antiviral agents which are so obviously needed, especially against the more important RNA viruses.

E. Expansion of the application of compounds with demonstrated antiviral activity against DNA viruses which are usually clinically silent or of minor importance in naturally occurring disease (e.g., herpes, zoster, cytomegalovirus) will probably be needed as increased use of immunosuppressive drugs as anticancer agents or as adjuvants to organ transplant therapy occurs. The application of pyrimidine antiviral agents useful against DNA viruses in such cases has been clearly demonstrated and should be expanded.

F. The arabinosyl nucleosides of both purines and pyrimidines are of great interest and potential utility. Ara-C is clearly of great interest and potential value as an antiviral drug in human medicine. Ara-A, as yet untested in man, also has great potential as an antiviral drug (Schabel, 1968). The biological history of these compounds and their promise has been reviewed by Cohen (1966) and their antiviral properties have been reviewed by De Rudder and Privat de Garilhe (1966b). The indication for intensive study of additional arabinosyl nucleosides (in addition to ara-C and ara-A) for antiviral activity is clear. Useful antiviral activity superior to that possessed by ara-C or promised by ara-A may be found.

G. IDU and ara-C are established examples that useful purine and/or pyrimidine antivirals can be synthesized or found in nature. If the development of effective antivirals mimics other successful drug research, promising laboratory demonstrated drug activity can be expanded by rational congener synthesis and useful drugs will ultimately be brought to practical utility in human medicine. The need for useful antivirals is great. That they can be developed has been demonstrated. The laboratory procedures and biological systems necessary to discover and develop new antiviral drugs to the point of clinical effectiveness are available. We only need the commitment to the effort to make great progress toward controlling one of the most important problems in human medicine.

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TABLE 1. CHEMICAL CLASSIFICATION OF PURINES AND PYRIMIDINES WHICH HAVE BEEN EXAMINED FOR ANTIVIRAL ACTIVITY

I. Purines
- A. 6-Aminopurines and Analogs
  1. Adenine and adenosine
  2. Substituted at the 2-carbon
  3. Substituted at the amino group
  4. Other nucleosides of adenine
  5. Ring analogs
- B. 6-Oxypurines and Analogs
  1. Hypoxanthine, xanthine, guanine and their natural nucleosides
  2. Other 6-oxypurines
  3. The 6-thiopurines
  4. Ring analogs
- C. Miscellaneous

II. Pyrimidines
- A. Uracil Derivatives
  1. Nucleosides of uracil
  2. 5-substituted uracils and their nucleosides
     a. 5-halo derivatives
     b. 5-alkyl derivatives
     c. Other 5-substituted derivatives
  3. N-alkyl derivatives of uracil
- B. Cytosine Derivatives
- C. 2-Thiopyrimidines
- D. Isocytosine and Alkyl Derivatives
- E. 6-Azapyrimidines
- F. Miscellaneous

TABLE 2. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF ADENINE AND ADENOSINE IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Adenine	Vaccinia (DNA)	Human	+	St. Geme (1969)
	Polio 2 (RNA)	Monkey	–	Knox *et al.* (1957)
Adenosine	Vaccinia (DNA)	Human	+	St. Geme (1969)
	Polio 2 (RNA)	Monkey	–	Knox *et al.* (1957)

TABLE 3. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF 6-AMINOPURINES SUBSTITUTED AT THE 2-CARBON IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Adenine, 2-amino-	Vaccinia (DNA)	Rodent	+	Balduzzi and Morgan (1964)
	Polio (RNA)	Human	+	Gifford *et al.* (1954)
	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
	Encephalomyocarditis (RNA)	Rodent	+	Balduzzi and Morgan (1964)
	Russian spring-summer encephalitis (RNA)	Rodent	+	Friend (1951)
Adenosine, 2-fluoro-	Cytomegalo (DNA)	Human	–	Sidwell *et al.* (1969a)

TABLE 4. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF 6-AMINOPURINES SUBSTITUTED ON THE AMINO GROUP IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Trimethylpurin-6-ylammonium chloride	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)

TABLE 5. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF OTHER NUCLEOSIDES OF ADENINE IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Adenine, 9-β-D-arabinofuranosyl- (ara-A)	Vaccinia (DNA)	Human	+	Privat de Garilhe and De Rudder (1964)
	Vaccinia (DNA)	Human	+	De Rudder and Privat de Garilhe (1966a)
	Vaccinia (DNA)	Human	+	Schabel (1968)
	Vaccinia (DNA)	Human	+	Miller *et al.* (1969)
		Monkey	+	
	Myxoma (DNA)	Rodent	+	Sidwell *et al.* (1970a)
	Herpes (DNA)	Human	+	Privat de Garilhe and De Rudder (1966a)
	Herpes (DNA)	Human	+	De Rudder and Privat de Garilhe (1966a)
	Herpes (DNA)	Human	+	Schabel (1968)
	Herpes (DNA)	Human	+	Miller *et al.* (1969)
	Herpes (DNA)	Human	+	Person *et al.* (1970)
	Herpes B, Marmoset (DNA)	Monkey	+	Schabel (1968)
		Human	+	
	Herpes B, Marmoset (DNA)	Monkey	+	Miller *et al.* (1969)
		Human		
	Pseudorabies (DNA)	Rodent	+	Sidwell *et al.* (1970a)
	Varicella-zoster (DNA)	Human	+	Schabel (1968)
	Varicella-zoster (DNA)	Human	+	Miller *et al.* (1969)
	Cytomegalo (DNA)	Human	+	Schabel (1968)

TABLE 5 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Adenine, 9-β-D-arabinofuranosyl- (ara-A) (continued)	Cytomegalo (DNA)	Human	+	Miller *et al.* (1969)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
	Adeno 3 (DNA)	Human	+	Schabel (1968)
	Adeno 3 (DNA)	Human	+	Miller *et al.* (1969)
	Polyoma (DNA)	Rodent	−	Schabel (1968)
	Polyoma (DNA)	Rodent	−	Miller *et al.* (1969)
	Polio 2 (RNA)	Monkey	−	Schabel (1968)
	Polio 2 (RNA)	Human	−	Miller *et al.* (1969)
	Coxsackie B1 (RNA)	Monkey	−	Schabel (1968)
	Coxsackie B1 (RNA)	Human	−	Miller *et al.* (1969)
	Echo-9 (RNA)	Monkey	−	Schabel (1968)
	Echo-9 (RNA)	Monkey	−	Miller *et al.* (1969)
	Rhino 1B (RNA)	Monkey	−	Schabel (1968)
	Rhino 1B (RNA)	Monkey	−	Miller *et al.* (1969)
	Newcastle (RNA)	Human	−	Schabel (1968)
	Newcastle (RNA)	Human	−	Miller *et al.* (1969)
	Parainfluenza 3 (RNA)	Human	−	Schabel (1968)
	Parainfluenza 3 (RNA)	Human	−	Miller *et al.* (1969)
	Measles (RNA)	Human	−	Schabel (1968)
	Measles (RNA)	Human	−	Miller *et al.* (1969)
	Respiratory syncytial (RNA)	Human	−	Schabel (1968)
	Respiratory syncytial (RNA)	Human	−	Miller *et al.* (1969)

TABLE 5 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Adenine, 9-β-L-arabinofuranosyl	Vaccinia (DNA)	Human	−	Miller *et al.* (1969)
	Herpes (DNA)	Human	−	Miller *et al.* (1969)
Adenine, 9-β-D-erythropenta-furanosyl-	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Adenosine, 5′-amino-5′-deoxy-	Herpes (DNA)	Monkey	+	Diwan *et al.* (1969)
Adenosine, 5′-amino-2′,5′-dideoxy-	Herpes (DNA)	Monkey	+	Diwan *et al.* (1969)
Adenosine, 3′-amino-3′-deoxy-*N*,*N*-dimethyl-	Encephalomyocarditis (RNA)	Rodent	−	Farnham (1965)
Adenosine, 3′-(α-amino-p-methoxy-hydrocinnamamido)-3′-deoxy-*N*,*N*-dimethyl-(Puromycin)	Herpes (DNA)	Human	+	Roizman (1963)
	Herpes (DNA)	Rodent	+	Newton (1965)
	Adeno 3, 7 (DNA)	Monkey	+	Mayor (1964)
	Polyoma (DNA)	Rodent	+	Bowen *et al.* (1966)
	SV40 (DNA)	Monkey	−	Melnick and Rapp (1965)
	SV40 (DNA)	Monkey	−	Rapp *et al.* (1965b)
	Polio 1 (RNA)	Human	+	Levintow *et al.* (1962)
	Polio 1 (RNA)	Human	+	Wecker (1963)
	Encephalomyocarditis (RNA)	Rodent	+	Farnham (1965)
	Encephalomyocarditis (RNA)	Rodent	+	Galegov and Kaverin (1967)
	Reo (RNA)	Rodent	+	Kudo and Graham (1966)
	Western Equine encephalitis (RNA)	Human	+	Wecker (1963)

TABLE 5 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Adenosine, 3′-(α-amino-p-methoxy-hydrocinnamamido)-3′-deoxy-*N*,*N*-dimethyl-(Puromycin) (continued)	Mouse hepatitis (RNA)	Porcine	+	Greig and Girard (1969)
	Transmissible gastroenteritis of swine (RNA)	Porcine	+	McClurkin and Norman (1967)
Adenosine, 2′-deoxy-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Adenosine, 5′-methylsulfonylamino	Herpes (DNA)	Monkey	+	Diwan *et al.* (1969)

TABLE 6. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF HYPOXANTHINE, XANTHINE, GUANINE AND THEIR NATURAL NUCLEOSIDES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Guanine	Vaccinia (DNA)	Human	–	St. Geme (1969)
	Polio 2 (RNA)	Monkey	–	Knox *et al.* (1957)
Guanosine	Vaccinia (DNA)	Human	–	St. Geme (1969)
	Polio 2 (RNA)	Monkey	–	Knox *et al.* (1957)
Guanosine, 2′-deoxy-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Guanylic acid	Polio 2 (RNA)	Monkey	–	Knox *et al.* (1957)
Hypoxanthine	Vaccinia (DNA)	Human	–	St. Geme (1969)
Inosine	Vaccinia (DNA)	Human	–	St. Geme (1969)
Xanthine	Vaccinia (DNA)	Human	–	St. Geme (1969)
	Polio 2 (RNA)	Monkey	–	Knox *et al.* (1957)

TABLE 7. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF OTHER 6-OXYPURINES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Caffeine	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
Hypoxanthine, 9-β-D-arabino-furanosyl-	Herpes (DNA)	Human	+	Miller *et al.* (1969)
Theobromine	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
Theophylline	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
Uric acid	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
Uric acid, 4,5-dihydroxy-	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
Xanthine, 8-chloro-	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)

TABLE 8. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF THE 6-THIOPURINES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Purine, 2-amino-6-(benzylthio)-	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine, 2-amino-6-[(1-methyl-4-nitroimidazol-5-yl)thio]-	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
Purine, 6-(benzylthio)-	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine, 6-[(1-methyl-4-nitro-imidazol-5-yl)thio]-	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine, 6-(methylthio)-	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine, 6-(methylthio)-9-β-D-ribofuranosyl-	Polyoma (DNA)	Rodent	+	Bowen *et al.* (1969)
	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine, 6-(propylthio)-	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
Purine-6(1H)-thione (6-mercaptopurine)	Vaccinia (DNA)	Human	+	St. Geme (1964)
	Vaccinia (DNA)	Human	+	St. Geme *et al.* (1969)
	Vaccinia (DNA)	Human	+	St. Geme (1969)
	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Herpes (DNA)	Human	+	St. Geme (1964)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
	Polio 1 (RNA)	Human	+	St. Geme (1964)
	Measles (RNA)	Human	+	St. Geme (1964)
Purine-6(1H)-thione, 2-amino- (thioguanine)	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)

TABLE 8 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Purine-6(1H)-thione, 2-amino-1-methyl-	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
Purine-6(1H)-thione, 2-amino-9-β-D-ribofuranosyl- (thioguanosine)	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
Purine-6(1H)-thione, 9-butyl	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine-6(1H)-thione, 9-cyclopentyl-	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine-6(1H)-thione, 9-ethyl	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)
Purine-6(1H)-thione, 9-β-D-ribofuranosyl-	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)
Purin-6-ylthioacetonitrile	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1969a)

TABLE 9. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF RING ANALOGS OF 6-OXYPURINES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
8-Azaguanine	Vaccinia (DNA)	Rodent	+	Balduzzi and Morgan (1964)
	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Adeno 4 (DNA)	Human	+	Starcheus and Chernetskii (1967)
	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)
	Mouse encephalomyelitis (RNA)	Rodent	−	Rafelson *et al.* (1950)
	Encephalomyocarditis (RNA)	Rodent	+	Balduzzi and Morgan (1964)
	Russian spring-summer encephalitis (RNA)	Rodent	−	Friend (1951)
	Measles (RNA)	Human	+	Hall *et al.* (1968)
8-Azaxanthine	Polio 2 (RNA)	Monkey	−	Knox *et al.* (1957)

TABLE 10. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF MISCELLANEOUS PURINE ANALOGS IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Purine, 8-bromo-	Cytomegalo (DNA)	Human	–	Sidwell *et al.* (1969a)
Purine, 6-chloro-	Cytomegalo (DNA)	Human	–	Sidwell *et al.* (1969a)
Purine, 6-chloro-9-β-D-ribofuranosyl-	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	–	Sidwell *et al.* (1969a)
Purine, 2-(methylthio)-9-β-D-ribofuranosyl-	Cytomegalo (DNA)	Human	–	Sidwell *et al.* (1969a)
Purine-6-carboxaldehyde thiosemicarbazone	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1969a)

TABLE 11. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF NUCLEOSIDES OF URACIL IN MAMMALIAN CELL CULTURE

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uracil, 1-β-D-arabinofuranosyl-	Vaccinia (DNA)	Human	–	De Rudder and Privat de Garilhe (1966a)
	Herpes (DNA)	Human	–	De Rudder and Privat de Garilhe (1966a)
	Polio 1 (RNA)	Human	–	De Rudder and Privat de Garilhe (1966a)
	Measles (RNA)	Human	–	De Rudder and Privat de Garilhe (1966a)
Uridine, 2′-deoxy-	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
	Mouse encephalomyelitis (RNA)	Rodent	–	Pearson *et al.* (1956)

TABLE 12. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF 5-HALOURACILS AND THEIR NUCLEOSIDES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uracil,1-β-D-arabinofuranosyl-5-iodo-	Vaccinia (DNA)	Human	+	Privat de Garilhe and De Rudder (1966a)
	Vaccinia (DNA)	Human	+	De Rudder and Privat de Garilhe (1966)
	Vaccinia (DNA)	Rodent	−	Renis *et al.* (1968)
	Vaccinia (DNA)	Rodent	+	Renis (1970)
	Herpes (DNA)	Human	+	Privat de Garilhe and De Rudder (1966a)
	Herpes (DNA)	Human	+	De Rudder and Privat de Garilhe (1966a)
	Herpes (DNA)	Rodent	+	Renis (1970)
	Pseudorabies (DNA)	Rodent	+	Renis *et al.* (1968)
	Pseudorabies (DNA)	Rodent	+	Renis (1970)
	Polio 1 (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
	Measles (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
Uracil, 5-bromo-	Vaccinia (DNA)	Human	−	Simon (1961)
	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	−	Sidwell *et al.* (1970b)
	Polio 3 (RNA)	Monkey Rodent Human	+	Li (1959)

TABLE 12 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uracil, 5-bromo- (continued)	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Uracil, 5-chloro-	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
	Mouse encephalomyelitis (RNA)	Rodent	–	Visser *et al.* (1952)
Uracil, 5-fluoro-	Vaccinia (DNA)	Human	+	Simon (1961)
	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
	Pseudorabies (DNA)	Rodent	+	Kaplan and Ben-Porat (1961)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
	Adeno (simian SV15) (DNA)	Rodent Monkey	–	Fong *et al.* (1968)
	SV40 (DNA)	Monkey	+	Melnick and Rapp (1965)
	SV40 (DNA)	Monkey	+	Rapp *et al.* (1965b)
	Polio (RNA)	Human	–	Simon (1961)
	Newcastle (RNA)	Human	–	Simon (1961)
Uracil, 5-iodo-	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
	Cytomegalo (DNA)	Human	–	Sidwell *et al.* (1970b)
Uridine, 5-bromo-	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
Uridine, 5-bromo-2′-deoxy-	Vaccinia (DNA)	Human	+	Simon (1961)
	Vaccinia (DNA)	Human	+	Easterbrook and Davern (1963)
	Vaccinia (DNA)	Rodent	+	Dubbs and Kit (1964a)
	Vaccinia (DNA)	Human	–	Furusawa *et al.* (1964)
	Vaccinia (DNA)	Monkey	+	Shen *et al.* (1966)

TABLE 12 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uridine, 5-bromo-2′-deoxy- (continued)	Vaccinia (DNA)	Human	+	Herrmann (1968)
	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Herpes (DNA)	Rodent	+	Kaufman and Maloney (1963a)
	Herpes (DNA)	Rodent	+	Dubbs and Kit (1964b)
	Herpes (DNA)	Human	+	Schneweis (1965)
	Herpes (DNA)	Monkey	+	Shen *et al.* (1966)
	Herpes (DNA)	Human	+	Herrmann (1968)
	Pseudorabies (DNA)	Rodent	−	Renis *et al.* (1968)
	Pseudorabies (DNA)	Porcine	+	Greig and Girard (1969)
	Herpes equine 3 (DNA)	Rodent	+	Karpas (1967)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
	African swine fever (DNA)	Porcine	+	Moulton and Coggins (1968)
	Adeno 5 (DNA)	Human	+	Kjellen (1962)
	Adeno 12 (DNA)	Human	−	Furusawa *et al.* (1964)
	Adeno 2 (DNA)	Monkey	+	Shen *et al.* (1966)
	Adeno, 1, 2, 5, 7 (DNA)	Human	−	Herrmann (1968)
	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970b)
	Polio (RNA)	Human	−	Simon (1961)
	Teschen disease-Talfan (porcine enterovirus) (RNA)	Porcine	−	Clarke (1968)
	Mouse encephalomyelitis (RNA)	Rodent	−	Pearson *et al.* (1956)
	Newcastle (RNA)	Human	−	Simon (1961)

TABLE 12 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uridine, 5-bromo-2′-deoxy- (continued)	Mouse hepatitis (HEV) (RNA)	Porcine	−	Greig and Girard (1969)
	Transmissible gastroenteritis (RNA)	Porcine	−	McClurkin and Norman (1967)
	Transmissible gastroenteritis (RNA)	Porcine	−	Clarke (1968)
	O Agent (RNA)	Monkey	−	Malherbe and Strickland-Cholmley (1967)
	Simian SA11 (RNA)	Monkey	−	Malherbe and Strickland-Cholmley (1967)
	Feline fibrosarcoma (RNA)	Canine	−	McKissick and Lamont (1970)
Uridine, 5-chloro-	Mouse encephalomyelitis (RNA)	Rodent	+	Rafelson *et al.* (1951)
	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
Uridine, 5-chloro-2′-deoxy-	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Vaccinia (DNA)	Human	+	Herrmann (1968)
	Herpes (DNA)	Rodent	+	Kaufman and Maloney (1963a)
	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Human	−	Herrmann (1968)
	Adeno 2 (DNA)	Monkey	±	Shen *et al.* (1966)
	Adeno 1, 2, 5, 7 (DNA)	Human	−	Herrmann (1968)
Uridine, 2′-deoxy-5-fluoro-	Vaccinia (DNA)	Human	+	Salzman (1960)
	Vaccinia (DNA)	Human	+	Nihoul and Van den Bulcke (1962)

TABLE 12 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uridine, 2′-deoxy-5-fluoro- (continued)	Vaccinia (DNA)	Human	+	Salzman (1962)
	Vaccinia (DNA)	Human	+	Salzman *et al.* (1963)
	Vaccinia (DNA)	Human	+	Shatkin and Salzman (1963)
	Vaccinia (DNA)	Monkey	+	Maassab and Cochran (1964)
	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Vaccinia (DNA)	Human	+	Henigst and Pielsticker (1968)
	Vaccinia (DNA)	Human	−	Herrmann (1968)
	Vaccinia (DNA)	Human	+	Umeda and Heidelberger (1969)
	Shope fibroma (DNA)	Rodent	+	Minocha and Maloney (1970)
	Sheep pox (DNA)	Ovine	+	Vigario *et al.* (1968)
	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Human	−	Herrmann (1968)
	Cytomegalo (DNA)	Human	+	Goodheart *et al.* (1963)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
	Malignant catarrhal fever (Group B herpes) (DNA)	Bovine	−	Plowright *et al.* (1963)
	African swine fever (cytoplasmic) (DNA)	Porcine	+	Moulton and Coggins (1968)
	Adeno, 4, 5 (DNA)	Human	+	Flanagan and Ginsberg (1962)
	Adeno 5 (DNA)	Human	+	Kjellen (1962)
	Adeno 2 (DNA)	Monkey	−	Shen *et al.* (1966)
	Adeno 1, 2, 5, 7 (DNA)	Human	−	Herrmann (1968)

TABLE 12 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uridine, 2′-deoxy-5-fluoro- (continued)	Infectious canine hepatitis (adeno) (DNA)	Canine	+	Moulton and Frazier (1963)
	Infectious canine hepatitis (adeno) (DNA)	Canine	+	Moulton and Zee (1969)
	Polyoma (DNA)	Rodent	+	Urbano (1967)
	Polyoma (DNA)	Rodent	+	Consigli *et al.* (1968)
	Polyoma (DNA)	Rodent	+	Pétursson and Weil (1968)
	SV40 (DNA)	Monkey	+	Rapp *et al.* (1965b)
	Polio (RNA)	Human	−	Salzman (1960)
	Polio (RNA)	Human	−	Salzman (1962)
	Polio (RNA)	Human	−	Salzman *et al.* (1963)
	Polio (RNA)	Monkey	−	Maassab and Cochran (1964)
	Echo-11 (RNA)	Monkey	−	Maassab and Cochran (1964)
	Corriparta (? arbovirus) (RNA)	Porcine	−	Carley and Standfast (1969)
	Rubella (RNA)	Monkey	−	Maassab and Cochran (1964)
Uridine, 2′-deoxy-5-iodo- (IDU)	Vaccinia (DNA)	Human	+	Loddo *et al.* (1963a)
	Vaccinia (DNA)	Human	+	Loddo *et al.* (1963b)
	Vaccinia (DNA)	Human	+	Rada and Blaškovič (1966)
	Vaccinia (DNA)	Monkey	+	Shen *et al.* (1966)
	Vaccinia (DNA)	Human	+	Herrmann (1968)
	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Vaccinia (DNA)	Human	+	Neufahrt *et al.* (1969)
	Vaccinia (DNA)	Human	+	Umeda and Heidelberger (1969)

TABLE 12 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Vaccinia (DNA)	Rodent	+	Renis (1970)
	Herpes (DNA)	Human	+	Cramer *et al.* (1963)
	Herpes (DNA)	Rodent	+	Kaufman and Maloney (1963a)
	Herpes (DNA)	Rodent	+	Dubbs and Kit (1964b)
	Herpes (DNA)	Monkey	+	Nemes and Hilleman (1965)
	Herpes (DNA)	Monkey	+	Shen *et al.* (1966)
	Herpes (DNA)	Human	+	Herrmann (1968)
	Herpes (DNA)	Human	+	Person *et al.* (1970)
	Herpes (DNA)	Rodent	+	Renis (1970)
	Herpes (DNA)	Human	+	Smith (1963)
		Rodent		
	Herpes (DNA)	Human	−	Smith and Dukes (1964)
	Herpes (simian B) (DNA)	Monkey	+	Miller (1967)
	Pseudorabies (DNA)	Rodent	+	Kaplan and Ben-Porat (1967)
	Pseudorabies (DNA)	Rodent	+	Renis *et al.* (1968)
	Pseudorabies (DNA)	Porcine	+	Greig and Girard (1969)
	Rhinotracheitis (DNA)	Bovine	+	Persechino and Orfei (1965)
	Rhinotracheitis (DNA)	Bovine	+	Lillie and Mohanty (1968)
	Varicella-zoster (DNA)	Human	−	Rapp and Vanderslice (1964)
	Varicella-zoster (DNA)	Human	+	Rawls *et al.* (1964)
	Cytomegalo (DNA)	Rodent	+	Henson *et al.* (1966)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
	Malignant catarrhal fever (group B herpes) (DNA)	Bovine	+	Plowright *et al.* (1963)

TABLE 12 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	African swine fever (DNA)	Porcine	+	Moulton and Coggins (1968)
	Adeno 2 (DNA)	Monkey	+	Shen *et al.* (1966)
	Adeno 1, 2, 5, 7 (DNA)	Human	−	Herrmann (1968)
	Polyoma (DNA)	Rodent	+	Munyon *et al.* (1964)
	Polyoma (DNA)	Rodent	+	Bowen *et al.* (1966)
	Polyoma (DNA)	Rodent	+	Kimura and Mori (1966)
	Polyoma (DNA)	Rodent	+	Kimura *et al.* (1966)
	SV40 (DNA)	Monkey	+	Melnick and Rapp (1965)
	SV40 (DNA)	Monkey	+	Rapp *et al.* (1965a)
	SV40 (DNA)	Monkey	+	Rapp *et al.* (1965b)
	"Porcine picodna" (PPV) (DNA)	Porcine	+	Mayr *et al.* (1968)
	Hamster osteolytic (DNA)	Monkey	+	Ledinko (1967)
	Polio 1 (RNA)	Human	−	Loddo *et al.* (1963b)
	Coxsackie B3 (RNA)	Human	−	Loddo *et al.* (1963b)
	Corriparta (? arbovirus) (RNA)	Porcine	−	Carley and Standfast (1969)
	Junin (RNA)	Monkey	−	Coto and De Vombergar (1969)
	Nariva (RNA)	Monkey	−	Karabatsos *et al.* (1969)
	Mutucare (RNA)	Monkey		Justines and Kuns (1970)
	Transmissible gastroenteritis (RNA)	Porcine	−	McClurkin and Norman (1967)
	Lymphocytic choriomeningitis (RNA)	Rodent	−	Furusawa *et al.* (1963)

TABLE 12 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Haemagglutinating encephalomyelitis (HEV) (RNA)	Porcine	−	Greig and Girard (1969)
	Simian SA11 (RNA)	Monkey	−	Malherbe and Strickland-Cholmley (1967)
	O Agent (RNA)	Monkey	−	Malherbe and Strickland-Cholmley (1967)
Uridine, 2′-deoxy-5-iodo, 5′-triphosphate-	Herpes (DNA)	Monkey	+	Diwan and Prusoff (1968)
Uridine, 5-fluoro-	Vaccinia (DNA)	Human	+	Nihoul and Van den Bulcke (1962)

TABLE 13. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF 5-ALKYLURACILS AND THEIR NUCLEOSIDES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Thymidine	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Thymine	Adeno 1, 12 (DNA)	Human	−	Muraoka *et al.* (1970a)
	Polio (RNA)	Human	−	Muraoka *et al.* (1970a)
Thymine, 1-β-D-arabinofuranosyl-	Vaccinia (DNA)	Human	+	De Rudder and Privat de Garilhe (1966a)
	Herpes (DNA)	Rodent	+	Underwood (1964)
	Herpes (DNA)	Rodent	+	Underwood *et al.* (1964)
	Herpes (DNA)	Human	+	De Rudder and Privat de Garilhe (1966a)
	Polio 1 (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
	Measles (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
Thymine, 1-(2-deoxy-β-D-*threo*-pentofuranosyl)-	Herpes (DNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
	Measles (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
Thymine, 1-β-D-glucopyranosyl-	Mouse encephalomyelitis (RNA)	Rodent	−	Visser *et al.* (1952)
Thymine, 1-β-D-ribofuranosyl-	Vaccinia (DNA)	Human	−	De Rudder and Privat de Garilhe (1966a)

TABLE 13 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Thymine,1-β-D-ribofuranosyl- (continued)	Herpes (DNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
	Polio 1 (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
	Measles (RNA)	Human	−	De Rudder and Privat de Garilhe (1966a)
Thymine, 1-β-D-ribopyranosyl-	Mouse encephalomyelitis (RNA)	Rodent	−	Visser *et al.* (1952)
Uracil, 5-butyl-	Vaccinia (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 1, 12 (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970b)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970b)
	Echo-28 (RNA)	Human	+	Muraoka *et al.* (1970a)
Uracil, 1-(2,3-dideoxy-2,3-didehydro-β-D-*glycero*-pentofuranosyl)-5-trifluoromethyl-	Vaccinia (DNA)	Human	−	Khwaja & Heidelberger (1969)
Uracil, 5-ethyl	Vaccinia (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 1,.12 (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970b)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970b)
	Echo-28 (RNA)	Human	+	Muraoka *et al.* (1970a)

TABLE 13 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Uracil, 5-ethyl-1-β-D-glucopyranosyl-	Adeno 1 (DNA)	Human	−	Muraoka *et al.* (1970a)
	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970b)
	Polio 1 (RNA)	Human	−	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970b)
	Echo-28 (RNA)	Human	+	Muraoka *et al.* (1970a)
Uracil, 5-hexyl-	Adeno 1, 12 (DNA)	Human	−	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	—	Muraoka *et al.* (1970a)
Uracil, 5-propyl-	Adeno 1, 12 (DNA)	Human	−	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	−	Muraoka *et al.* (1970a)
Uridine, 5-butyl-	Vaccinia (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 1, 12 (DNA)	Human	+	Muraoka *et al.* (1970a)
	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970b)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970b)
	Echo-28 (RNA)	Human	+	Muraoka *et al.* (1970a)
Uridine, 2′-deoxy-5-ethyl-	Vaccinia (DNA)	Human	+	Swierkowski and Shugar (1969)
Uridine, 2′-deoxy-5-trifluoromethyl-	Vaccinia (DNA)	Monkey	+	Shen *et al.* (1966)
	Vaccinia (DNA)	Human	+	Umeda and Heidelberger (1969)
	Vaccinia (DNA)	Human	+	Fujiwara and Heidelberger (1970)
	Herpes (DNA)	Monkey	+	Shen *et al.* (1966)
	Adeno 2 (DNA)	Monkey	+	Shen *et al.* (1966)
Uridine, 5-ethyl	Adeno 1, 12 (DNA)	Human	−	Muraoka *et al.* (1970a)

TABLE 13 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uridine, 5-ethyl- (continued)	Adeno 12 (DNA)	Human	+	Muraoka *et al.* (1970b)
	Polio 1 (RNA)	Human	−	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	+	Muraoka *et al.* (1970b)
Uridine, 6-methyl-	Herpes (DNA)	Monkey	+	Diwan *et al.* (1969)
Uridine, 5-trifluoromethyl-	Vesicular stomatitis (RNA)	Rodent	−	Khwaja and Heidelberger (1969)

TABLE 14. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF OTHER 5-SUBSTITUTED URACILS AND THEIR NUCLEOSIDES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uracil, 5-amino-	Adeno (DNA)	Monkey Human	−	Hollinshead (1962)
	Polio (RNA)	Monkey Human	−	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	−	Hollinshead (1962)
Uracil, 5-diazo-	Adeno (DNA)	Monkey Human	−	Hollinshead (1962)
	Polio	Monkey Human	−	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	−	Hollinshead (1962)
Uracil, 5-methylamino-	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
	Adeno 2 (DNA)	Monkey	−	Shen *et al.* (1966)
Uridine, 5-acetylamino-3′,5′-di-*O*-acetyl-2′-deoxy-	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
Uridine, 5-amino-	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
Uridine, 5-amino-2′-deoxy-, hydrochloride	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
Uridine, 2′-deoxy-5-dimethylamino-	Herpes (DNA)	Monkey	+	Shen *et al.* (1966)

TABLE 14 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uridine, 2′-deoxy-5-ethylamino-	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Monkey	+	Shen *et al.* (1966)
	Adeno 2 (DNA)	Monkey	−	Shen *et al.* (1966)
Uridine, 2′-deoxy-5-hydroxy-	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
	Adeno 2 (DNA)	Monkey	−	Shen *et al.* (1966)
	Mouse encephalomyelitis (RNA)	Rodent	−	Pearson *et al.* (1956)
Uridine, 2′-deoxy-5-methylamino-	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Monkey	+	Nemes and Hilleman (1965)
	Herpes (DNA)	Monkey	+	Shen *et al.* (1966)
	Adeno 2 (DNA)	Monkey	−	Shen *et al.* (1966)
	Coxsackie B2 (RNA)	Monkey	−	Shen *et al.* (1966)
	Parainfluenza (RNA)	Monkey	−	Shen *et al.* (1966)
Uridine, 5-diazo-	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
Uridine, 5-diazo-2′-deoxy-	Vaccinia (DNA)	Monkey	−	Shen *et al.* (1966)
	Herpes (DNA)	Monkey	−	Shen *et al.* (1966)
	Adeno 2 (DNA)	Monkey	−	Shen *et al.* (1966)
Uridine, 5-formamido-	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
Uridine, 5-hydroxy-	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)
	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)

TABLE 15. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF N-ALKYLURACILS AND THEIR NUCLEOSIDES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uracil, 1-β-D-arabinofuranosyl-3-methyl-	Herpes (DNA)	Rodent	–	Renis *et al.* (1968)
Uracil, 3-butyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3-butyl-6-methyl-	Adeno 1, 12 (RNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3,6-dimethyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3-ethyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3-ethyl-6-methyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3-hexyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3-methyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uracil, 3-methyl-6-propyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Uridine, 3-methyl-	Mouse encephalomyelitis (RNA)	Rodent	+	Visser *et al.* (1952)

TABLE 16. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF CYTOSINE DERIVATIVES IN MAMMALIAN CELL CULTURE

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Adenosine, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Adenosine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Adenosine, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Adenosine, 2′-deoxy-, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Adenosine, 2′-deoxy-, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, adenylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, adenylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)

TABLE 16 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Aracytidine, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, deoxyuridylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, thymidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, uridylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Aracytidine, uridylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Azauridine, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)

TABLE 16 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Cytidine	Herpes (DNA)	Rodent	−	Renis *et al.* (1968)
Cytidine, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Cytidine, 5-bromo-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Cytidine, 5-bromo-2′-deoxy-	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
Cytidine, 5-chloro-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Cytidine, 2′-deoxy-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Cytidine, 2′-deoxy-, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Cytidine, 2′-deoxy-5-fluoro-	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
Cytidine, 2′-deoxy-5-iodo-	Vaccinia (DNA)	Rodent	+	Renis (1970)
	Herpes (DNA)	Rodent	+	Renis (1970)
	Pseudorabies (DNA)	Rodent	+	Renis (1970)
	Cytomegalo (DNA)	Human	+	Sidwell *et al.* (1970b)
Cytidine, 2′-deoxy-3-methyl-, methylhydrogen sulfate	Herpes (DNA)	Rodent	−	Renis *et al.* (1968)

TABLE 16 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Cytidine, 2′-deoxy-, 5′-phosphate	Mouse encephalomyelitis (RNA)	Rodent	−	Pearson *et al.* (1956)
Cytidine, 5-hydroxy-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Cytidine, 6-methyl-	Herpes (DNA)	Monkey	+	Diwan *et al.* (1969)
Cytidine, 3-methyl-, methylhydrogen sulfate	Herpes (DNA)	Rodent	−	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl- (ara-C)	Vaccinia (DNA)	Rodent	+	Renis and Johnson (1962)
	Vaccinia (DNA)	Rodent Human	+	Buthala (1964)
	Vaccinia (DNA)	Rodent	+	Silagi (1965)
	Vaccinia (DNA)	Human	+	Herrmann (1968)
	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Vaccinia (DNA)	Human	+	Umeda and Heidelberger (1969)
	Vaccinia (DNA)	Monkey	+	Prince *et al.* (1969)
	Shope fibroma (DNA)	Rodent	+	Minocha and Maloney (1970)
	Herpes (DNA)	Rodent Human	+	Buthala (1964)
	Herpes (DNA)	Monkey Human	+	Levitt and Becker (1967)
	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Herpes (DNA)	Rodent	+	Ben-Porat *et al.* (1968)
	Herpes (DNA)	Rodent	+	Campbell *et al.* (1968)

TABLE 16 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Cytosine, 1-β-D-arabinofuranosyl- (ara-C) (continued)	Herpes (DNA)	Human	+	Herrmann (1968)
	Herpes (DNA)	Rodent	+	Renis *et al.* (1968)
	Herpes B (simian) (DNA)	Rodent	+	Buthala (1964)
	Pseudorabies (DNA)	Rodent	+	Buthala (1964)
	Pseudorabies (DNA)	Rodent	+	Ben-Porat *et al.* (1968)
	Pseudorabies (DNA)	Rodent	+	Renis *et al.* (1968)
	Pseudorabies (DNA)	Rodent	+	Ben-Porat *et al.* (1969)
	Adeno 2, 3, 6, 8, 10 (DNA)	Human	−	Buthala (1964)
	Adeno 2, 7, 12 (DNA)	Human	+	Feldman and Rapp (1966)
	Adeno SV15 (DNA)	Rodent Monkey	−	Fong *et al.* (1968)
	Adeno 1, 2, 5, 7 (DNA)	Human	−	Herrmann (1968)
	SV40 (DNA)	Monkey	+	Melnick and Rapp (1965)
	SV40 (DNA)	Monkey	+	Rapp *et al.* (1965a)
	SV40 (DNA)	Monkey	+	Rapp *et al.* (1965b)
	Hamster osteolytic (H-1) (DNA)	Monkey	+	Ledinko (1967)
	Coxsackie A21 (RNA)	Human	−	Buthala (1964)
	Coxsackie A21 (RNA)	Human	−	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Human	−	Renis *et al.* (1968)
	Coxsackie A21 (RNA)	Monkey	—	Prince *et al.* (1969)
	Encephalomyocarditis (RNA)	Monkey	−	Prince *et al.* (1969)

TABLE 16 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Cytosine, 1-β-D-arabinofuranosyl- (ara-C) (continued)	Mengo (RNA)	Rodent	−	Campbell *et al.* (1968)
	Reo (RNA)	Rodent	−	Silagi (1965)
	Reo 1 (RNA)	Rodent	−	Campbell *et al.* (1968)
	Sindbis (RNA)	Rodent	−	Campbell *et al.* (1968)
	Western equine Encephalomyelitis (RNA)	Rodent	−	Campbell *et al.* (1968)
	West Nile (RNA)	Rodent	−	Campbell *et al.* (1968)
	Vesicular stomatitis (Indiana) (RNA)	Rodent	+	Campbell *et al.* (1968)
	Vesicular stomatitis (New Jersey) (RNA)	Rodent	+	Campbell *et al.* (1968)
	Influenza A (RNA)	Rodent	−	Renis and Johnson (1962)
	Influenza A (RNA)	Monkey	−	Prince *et al.* (1969)
	Newcastle (RNA)	Rodent	−	Renis and Johnson (1962)
	Newcastle (RNA)	Rodent	−	Campbell *et al.* (1968)
	Parainfluenza 3 (RNA)	Human	−	Buthala (1964)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Rodent	−	Renis *et al.* (1968)
	Rabies (RNA)	Rodent Human	+	Campbell *et al.* (1968)
	Cocal (RNA)	Rodent	−	Campbell *et al.* (1968)
	Moloney murine sarcoma (RNA)	Rodent	+	Hirschman *et al.* (1969)

TABLE 16 (*cont.*)

Compound	Virus Name (Type)	Cell culture	Activity	Reference
Cytosine, 1-β-D-arabinofuranosyl- (ara-C) (continued)	Harvey murine sarcoma (RNA)	Rodent	+	Murray and Temin (1970)
	Lymphocytic choriomeningitis (RNA)	Rodent	−	Campbell *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-5-bromo-	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Herpes (DNA)	Rodent	+	Renis *et al.* (1968)
	Pseudorabies (DNA)	Rodent	+	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-5-chloro-	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Herpes (DNA)	Rodent	+	Renis *et al.* (1968)
	Pseudorabies (DNA)	Rodent	+	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-5-fluoro-	Coxsackie B1 (RNA)	Monkey	−	Prince *et al.* (1969)
	Encephalomyocarditis (Columbia SK) (RNA)	Monkey	−	Prince *et al.* (1969)
	Influenza A (RNA)	Monkey	−	Prince *et al.* (1969)
Cytosine, 1-β-D-arabinofuranosyl-5-iodo-	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Vaccinia (DNA)	Rodent	+	Renis (1970)
	Herpes (DNA)	Rodent	+	Renis *et al.* (1968)
	Herpes (DNA)	Rodent	+	Renis (1970)
	Pseudorabies (DNA)	Rodent	−	Renis *et al.* (1968)
	Pseudorabies (DNA)	Rodent	+	Renis (1970)

TABLE 16 (*cont.*)

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Cytosine, 1-β-D-arabinofuranosyl-5-methyl-	Herpes (DNA)	Rodent	−	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-3-methyl-, methylhydrogen sulfate	Vaccinia (DNA)	Rodent	+	Renis *et al.* (1968)
	Herpes (DNA)	Rodent	+	Renis *et al.* (1968)
	Pseudorabies (DNA)	Rodent	−	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-, 2′-phosphate	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Cytosine, 1-β-D-arabinofuranosyl-, 3′-phosphate	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Cytosine, 1-β-D-arabinofuranosyl-, 5′-phosphate	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	−	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	−	Renis *et al.* (1967)
Cytosine, 5,6-diamino-	Adeno (DNA)	Monkey Human	−	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	−	Hollinshead (1962)
	Polio (RNA)	Monkey Human	−	Hollinshead (1962)

TABLE 16 (*cont.*)

Compound	Virus Name (Type)	Cell culture	Activity	Reference
Cytosine, 1-β-D-xylofuranosyl-, hydrochloride	Herpes (DNA)	Rodent	–	Renis *et al.* (1968)
Cytosine, 1-β-D-xylofuranosyl-3-methyl-, methylhydrogen sulfate	Herpes (DNA)	Rodent	–	Renis *et al.* (1968)
Cytosine, 5-methyl-, hydrochloride	Herpes (DNA)	Rodent	–	Kaufman and Maloney (1963a)
Cytosine, 3-methyl-1-β-D-xylofuranosyl-, hydrochloride	Herpes (DNA)	Rodent	–	Renis *et al.* (1968)
Cytosine, 5-methyl-1-β-D-xylofuranosyl-, methylhydrogen sulfate	Herpes (DNA)	Rodent	–	Renis *et al.* (1968)
Cytosine, 1-β-D-xylofuranosyl-, hydrochloride	Herpes (DNA)	Rodent	–	Renis *et al.* (1968)
Thymidine, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	–	Renis *et al.* (1967)
Thymidine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	–	Renis *et al.* (1967)
Uridine, aracytidylyl-[2′ → 5′]-	Herpes Simplex (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	–	Renis *et al.* (1967)
Uridine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	–	Renis *et al.* (1967)

TABLE 16 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Uridine, 2′-deoxy-, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	–	Renis *et al.* (1967)
Uridine, 2′-deoxy-, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza 3 (RNA)	Human	–	Renis *et al.* (1967)
Uridine, 2′-deoxy-, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rodent	+	Renis *et al.* (1967)
	Coxsackie A21 (RNA)	Rodent	–	Renis *et al.* (1967)
	Parainfluenza (RNA)	Human	–	Renis *et al.* (1967)

TABLE 17. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF 2-THIOPYRIMIDINES IN MAMMALIAN CELL CULTURE

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Cytosine, 6-amino-5-nitro-2-thio-	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)
Cytosine, 6-amino-2-thio-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Pyrimidine, 4-hydroxy-2-propylthio-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
5-Pyrimidinecarboxylic acid, 4-methyl-2-(methylthio)-, ethyl ester	Polio 2 (RNA)	Monkey	+	Yamazi *et al.* (1970)
	Echo 1, 5, 13, 22, 23 (RNA)	Monkey	−	Yamazi *et al.* (1970)
	Coxsackie A7, A9, B3, B5 (RNA)	Monkey	−	Yamazi *et al.* (1970)
Thymine, 2-thio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)
	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Uracil, 6-methyl-2-thio-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)
Uracil, 6-phenyl-2-thio-	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)
Uracil, 2-thio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)
	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)

TABLE 18. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF ISOCYTOSINE AND VARIOUS ALKYL DERIVATIVES IN MAMMALIAN CELL CULTURE

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Isocytosine	Adeno (DNA)	Monkey Human	–	Hollinshead (1962)
	Polio (RNA)	Monkey Human	–	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	–	Hollinshead (1962)
Isocytosine, 5-butyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-butyl-6-methyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5,6-dimethyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-ethyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-ethyl-6-methyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 6-hexyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-hexyl-6-methyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-methyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)

TABLE 18 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Isocytosine, 6-methyl-5-octyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 6-methyl-5-propyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-octyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)
Isocytosine, 5-propyl-	Adeno 1, 12 (DNA)	Human	–	Muraoka *et al.* (1970a)
	Polio 1 (RNA)	Human	–	Muraoka *et al.* (1970a)

TABLE 19. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF 6-AZAPYRIMIDINES IN MAMMALIAN CELL CULTURE

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
6-Azacytidine	Adeno 4 (DNA)	Human	+	Starcheus and Chernetskii (1967)
6-Azathymine, 2-thio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 1,3-dimethyl-2-thio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 1,3-dimethyl-4-thio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 2,4-dithio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
6-Azauracil, 1-methyl-2,4-dithio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
6-Azauracil, 3-methyl-2,4-dithio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 1-methyl-2-thio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 1-methyl-4-thio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
6-Azauracil, 3-methyl-2-thio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 3-methyl-4-thio-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
6-Azauracil, 2-thio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
6-Azauracil, 4-thio-	Vaccinia (DNA)	Monkey	−	Šmejkal *et al.* (1962)
6-Azauridine	Vaccinia (DNA)	Human	+	Loh (1964)
	Vaccinia (DNA)	Human	+	Rada and Blaškovič (1966)
	Herpes (DNA)	Rodent	−	Kaufman and Maloney (1963a)
	Herpes (DNA)	Human	+	Galegov *et al.* (1968)
	Herpes (DNA)	Rodent	+	Falke and Rada (1970)
	Cytomegalo (DNA)	Human	+	Demidova *et al.* (1969)
	Adeno 4 (DNA)	Human	+	Starcheus and Chernetskii (1967)
	Reo (RNA)	Rodent	+	Rada and Shatkin (1967)
	Lymphocytic choriomeningitis (RNA)	Rodent	+	Buck and Pfau (1969)
ar-Triazine, 3,5-di(methylthio)-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)

TABLE 19 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
as-Triazin-5(4H)-one, 4,6-dimethyl-3-(methylthio)-	Vaccinia (DNA)	Monkey	–	Šmejkal *et al.* (1962)
as-Triazin-3 (2H)-one, 2-methyl-5-(methylthio)-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
as-Triazin-5(2H)-one, 2-methyl-3-(methylthio)-	Vaccinia (DNA)	Monkey	–	Šmejkal *et al.* (1962)
as-Triazin-5(4H)-one, 6-methyl-3-(methylthio)-	Vaccinia (DNA)	Monkey	–	Šmejkal *et al.* (1962)
as-Triazin-5(4H)-one, 3-(methylthio)-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)
as-Triazin-5(4H)-thione, 4-methyl-3-(methylthio)-	Vaccinia (DNA)	Monkey	+	Šmejkal *et al.* (1962)

TABLE 20. REPORTED TESTING FOR *IN VITRO* ANTIVIRAL ACTIVITY OF MISCELLANEOUS PYRIMIDINES IN MAMMALIAN CELL CULTURE

Compound	Virus: Name (Type)	Cell culture	Activity	Reference
Alloxan	Adeno (DNA)	Monkey Human	−	Hollinshead (1962)
	Polio (RNA)	Monkey Human	−	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	−	Hollinshead (1962)
Barbituric acid, 5-allyl-5-pentadecyl-2-thio-	Vaccinia (DNA)	Human	−	Mosimann (1966)
	Polio 1, 2, 3 (RNA)	Human	−	Mosimann (1966)
	Echo 4, 6, 9 (RNA)	Human	−	Mosimann (1966)
Barbituric acid, 5-butyl-5-ethyl-	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)
Barbituric acid, 5-ethyl-5-(3′,4′-dichlorophenyl)-	Coxsackie A21 (RNA)	Human	+	DeLong *et al.* (1970)
Barbituric acid, 1-methyl-5-butyl-5-ethyl-	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)
Pyrimidine, 6-amino-2,4-dimethyl-	Adeno (DNA)	Monkey Human	−	Hollinshead (1962)
	Polio (RNA)	Monkey Human	−	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	−	Hollinshead (1962)
Pyrimidine, 2-amino-4-methyl-	Mouse encephalomyelitis (RNA)	Rodent	+	Pearson *et al.* (1956)

TABLE 20 (*cont.*)

Compound	Virus	Cell culture	Activity	Reference
	Name (Type)			
Pyrimidine, 5-butyl-4,6-dihydroxy-2-methyl-	Adeno (DNA)	Monkey Human	–	Hollinshead (1962)
	Polio (RNA)	Monkey Human	–	Hollinshead (1962)
	Influenza (RNA)	Monkey Human	–	Hollinshead (1962)
Pyrimidine, 2,4-dichloro-	Mouse encephalomyelitis (RNA)	Rodent	–	Pearson *et al.* (1956)
Pyrimidine, 2,4-dichloro-6-methyl-	Mouse encephalomyelitis (RNA)	Rodent	–	Pearson *et al.* (1956)
Pyrimidine, 2,4,5,6-tetraamino-	Polio 2 (RNA)	Monkey	+	Knox *et al.* (1957)

TABLE 21. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF ADENINE SULFATE

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Adenine sulfate	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	–	Thompson and Lavender (1953)

TABLE 22. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 6-AMINOPURINES SUBSTITUTED AT THE 2-CARBON

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Adenine, 2-amino-	Vaccinia (DNA)	Mice	i.c. i.n.	i.p. diet	−	Thompson *et al.* (1950)
	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
	Russian spring-summer encephalitis (RNA)	Mice	i.p.	i.p.	+	Moore and Friend (1951)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Adenine, 2-mercapto-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
Adenosine, 2-amino-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Adenosine, 2-fluoro-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)

TABLE 23. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 6-AMINOPURINES SUBSTITUTED AT THE AMINO GROUP

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Adenine, N-decyl-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
Adenosine, N-amino-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Purine, 6-(2,2-dimethyl-hydrazino)-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)

TABLE 24. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF NUCLEOSIDES OF ADENINE

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Adenine, 9-β-D-arabinofuranosyl- (ara-A)	Vaccinia (DNA)	Mice	i.c.	i.p., p.o., p.c.	+	Schabel (1968)
	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Vaccinia (DNA)	Mice	i.c.	i.p., p.o.	+	Dixon *et al.* (1969)
	Herpes (DNA)	Mice	i.c.	i.p., p.o., p.c.	+	Schabel (1968)
	Herpes (DNA)	Hamsters	cornea	topical	+	Schabel (1968)
	Herpes (DNA)	Mice	i.c.	i.p., s.c., p.o., p.c.	+	Sloan *et al.* (1969)
	Herpes (DNA)	Hamsters	cornea	topical	+	Sidwell (1969b)
	Herpes (DNA)	Hamsters	cornea	topical	+	Schardein & Sidwell (1969)
	Herpes (DNA)	Rabbits	cornea, intraocular	topical, s.c.	+	Kaufman *et al.* (1970)
	Herpes (DNA)	Owl monkeys	intraocular	s.c.	+	Kaufman *et al.* (1970)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Adenosine, 2'-C-methyl-	Vaccinia (DNA)	Mice	i.v.	i.p.	+	Walton *et al.* (1969)
Adenosine, 3'-C-methyl-	Vaccinia (DNA)	Mice	i.v.	i.p.	+	Walton *et al.* (1969)

TABLE 25. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF RING ANALOGS OF ADENINE AND ADENOSINE

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Pyrazolo(3,4-*d*)pyrimidine, 4-amino-	Vaccinia (DNA)	Mice	i.c.	i.p.	–	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	–	Sidwell *et al.* (1968a)
Pyrrolo(2,3-*d*)pyrimidine, 4-amino-7-β-D-ribofuranosyl- (Tubercidin)	Vaccinia (DNA)	Mice	i.c.	i.p.	–	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	–	Sidwell *et al.* (1968a)
Tetrazolo(1,5-*c*)pyrimidine, 8-amino-7-chloro-	Vaccinia (DNA)	Mice	i.c.	i.p.	–	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	–	Sidwell *et al.* (1968a)
ν-Triazolo(4,5-*d*)pyrimidine, 5,7-diamino-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	–	Thompson and Lavender (1953)

TABLE 26. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF DERIVATIVES OF 6-OXYPURINES

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Hypoxanthine, 9-β-D-arabinofuranosyl-	Herpes (DNA)	Mice	i.c.	i.p.	+	Schabel (1968)
Hypoxanthine, 8-mercapto-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)

TABLE 27. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 6-THIOPURINES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Purine, 2-amino-6-(benzylthio)-9-β-D-ribofuranosyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Purine, 2-amino-6, 8-dimercapto-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
Purine, 6-benzylthio-9-β-D-ribofuranosyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)
Purine, 2,6-dimercapto-8-hydroxy-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	− −	Thompson and Lavender (1953)
Purine, 1,3-dimethyl-2,6-dithio-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
Purine, 6-(ethylthio)-9-β-D-ribofuranosyl-, hydrate	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Purine,6-[(1-methyl-4-nitroimidazol-5-yl)thio]-, hydrate	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)
Purine, 6-methylthio-9-β-D-ribofuranosyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)

TABLE 27 (*cont.*)

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Purine-6(1H)-thione (6-mercaptopurine)	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Vaccinia (DNA)	Mice	i.p.	i.p.	−	St. Geme *et al.* (1970)
	Herpes (DNA)	Mice	i.p.	i.p.	−	St. Geme *et al.* (1968)
	Herpes (DNA)	Mice	i.p.	i.p.	−	St. Geme *et al.* (1970)
	Coxsackie B3, 5 (RNA)	Mice	i.p.	i.p.	−	St. Geme *et al.* (1970)
	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	s.c.	+	Ebert *et al.* (1968)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)
	Friend leukemia (RNA)	Mice	i.p.	i.p. s.c.	+	Sidwell *et al.* (1969c)
	Lactate dehydrogenase (RNA)	Mice	i.p.	s.c.	−	Ebert *et al.* (1968)
Purine-6(1H)-thione, 2-amino- (thioguanine)	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
	Influenza A (RNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)

TABLE 27 (*cont.*)

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Purine-6(1H)-thione, 2-amino-9-β-D-ribofuranosyl- (thioguanosine)	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)
Purine-6(1H)-thione, 9-butyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)
Purine-6(1H)-thione, 9-cyclopentyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)
Purine-6(1H)-thione, 9-β-D-ribofuranosyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1966)
	Friend leukemia (RNA)	Mice	i.p.	i.p.	+	Sidwell *et al.* (1968b)

TABLE 28. REPORTED *IN VIVO* TESTING FOR ANTIVIRAL ACTIVITY OF RING ANALOGS OF 6-OXYPURINES

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
8-Azaguanine	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Lymphocytic choriomeningitis (RNA)	Mice	i.c.	i.p.	+	Haas and Stewart (1956)
Pyrazolo-(3,4-*d*)-pyrimidin-4(5H)-one	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)

TABLE 29. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF MISCELLANEOUS PURINES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Purine, 8-(benzylthio)-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Purine, 6-chloro-9-β-D-ribofuranosyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Purine, 2,6-dichloro-7-methyl-	Vaccinia (DNA)	Mice	i.c. i.n.	i.p. diet	−	Thompson *et al.* (1950)
Purine, 6-methyl-	Semliki Forest (RNA)	Mice	i.c. i.p.	i.p. diet	−	Thompson and Lavender (1953)
Purine, 6-methyl-, 1-oxide	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Purine-6-carboxaldehyde thiosemicarbazone	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
1-Purin-6-ylsemicarbazide, 3-thio-, hydrate	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)

TABLE 30. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 1-β-D-ARABINOFURANOSYLURACIL

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Uracil, 1-β-D-arabino-furanosyl-	Herpes (DNA)	Rabbit	cornea	topical	–	Underwood (1964)
	Herpes (DNA)	Rabbit	cornea	topical	–	Underwood *et al.* (1964)

TABLE 31. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 5-HALOURACILS AND THEIR NUCLEOSIDES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Uracil, 1-β-D-arabino-furanosyl-5-bromo-	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
Uracil, 1-β-D-arabino-furanosyl-5-chloro-	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
Uracil, 1-β-D-arabino-furanosyl-5-iodo	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
Uracil, 5-bromo-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Uracil, 5-fluoro-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	−	Kaufman *et al.* (1962c)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Lymphocytic choriomeningitis (RNA)	Mice	i.c.	s.c.	+	Levy and Haas (1958)
Uracil, 5-iodo-	Herpes (DNA)	Rabbits	cornea	topical	−	Kaufman *et al.* (1962c)
	Herpes (DNA)	Rabbits	cornea	topical	−	Hanna (1966)
Uracil, 1-(2,3,5-tri-*O*-acetyl-β-D-arabinofuranosyl)-5-chloro-	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
Uridine, 5-bromo-2'-deoxy-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman *et al.* (1962c)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman (1965b)

TABLE 31 (*cont.*)

Compound	Virus: Name (Type)	Host	Roate of virus inoc.	Route of R_x	Activity	Reference
Uridine, 5-bromo-2′-deoxy- (continued)	Herpes (DNA)	Rabbits	cornea	topical	+	Hanna (1966)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Uridine, 5-chloro-2′-deoxy-	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman *et al.* (1962c)
Uridine, 2′-deoxy-3′,5′-*O*-diacetyl-5-iodo-	Herpes (DNA)	Rabbits	cornea	topical	+	Perkins *et al.* (1962)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hanna (1966)
Uridine, 2′-deoxy-5-fluoro-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	−	Kaufman *et al.* (1962c)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Uridine, 2′-deoxy-5-iodo-	Vaccinia (DNA)	Rabbits	intra-dermal	s.c.	+	Calabresi *et al.* (1962)
	Vaccinia (DNA)	Rabbits	cornea	topical	+	Kaufman *et al.* (1962b)
	Vaccinia (DNA)	Rabbits	intra-dermal	s.c.	+	Calabresi (1963)
	Vaccinia (DNA)	Rabbits	intra-dermal	s.c.	+	Calabresi *et al.* (1963)
	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Ectromelia (DNA)	Mice	i.p.	s.c.	−	Furusawa *et al.* (1963)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman (1962)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman *et al.* (1962c)
	Herpes (DNA)	Rabbits	cornea	topical	+	Perkins *et al.* (1962)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1962)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman and Maloney (1963b)

TABLE 31 (*cont.*)

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Uridine, 2'-deoxy-5-iodo- (continued)	Herpes (DNA)	Monkeys	cornea	topical	+	Kaufman and Maloney (1963b)
	Herpes (DNA)	Rabbits	cornea	topical	+	Laibson *et al.* (1963)
	Herpes (DNA)	Rabbits	cornea	topical	+	Nemes and Hilleman (1965)
	Herpes (DNA)	Rabbits	cornea	topical	+	Engle and Stewart (1964)
	Herpes (DNA)	Rabbits	intra-dermal	s.c.	+	Force *et al.* (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman (1965b)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hanna and Wilkenson (1965)
	Herpes (DNA)	Rabbits	cornea	topical	+	Jawetz *et al.* (1965)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hanna (1966)
	Herpes (DNA)	Hamster	cornea	topical	+	Sidwell *et al.* (1966)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hyndiuk and Kaufman (1967)
	Herpes (DNA)	Mice	i.c.	i.p.	−	Schabel (1968)
	Herpes (DNA)	Hamsters	cornea	topical	+	Schabel (1968)
	Herpes (DNA)	Guinea pig	skin vaccina-tion	topical s.c.	+	Tomlinson and MacCallum (1968)
	Herpes (DNA)	Rabbits	cornea	topical	+	Pollikoff *et al.* (1970)
	Herpes (simian B) (DNA)	Rabbits	cornea	topical	−	Benda (1965)
	Pseudorabies (DNA)	Rabbits	cornea	topical	+	Kolb *et al.* (1963)

TABLE 31 (*cont.*)

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Uridine, 2'-deoxy-5-iodo- (continued)	Rhinotracheitis (DNA)	Rabbits	cornea, abdominal scarification	topical	+	Persechino and Orfei (1965)
	Adeno 12 (DNA)	Hamsters	s.c.	s.c.	+	Huebner *et al.* (1963)
	Encephalomyocarditis (Columbia SK) (RNA)	Mice	i.p.	s.c.	+	Force and Stewart (1964)
	Encephalomyocarditis (Columbia SK) (RNA)	Mice	i.p.	p.o.	−	Furusawa *et al.* (1963)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Uridine, 5-fluoro-2'-C-methyl-	Vaccinia (DNA)	Mice	i.v.	i.p.	−	Walton *et al.* (1969)

TABLE 32. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 5-ALKYLURACILS AND THEIR NUCLEOSIDES

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Thymine, 1-β-D-arabino-furanosyl-	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
Uridine, 2′-deoxy-5-ethyl-	Herpes (DNA)	Rabbits	cornea	topical	+	Gauri and Malorny (1967)
	Herpes (DNA)	Rabbits	cornea	topical	+	Gauri (1968)
	Herpes (DNA)	Rabbits	cornea	topical	+	Riehm and Gauri (1969)
Uridine, 2′-deoxy-5-propyl-	Herpes (DNA)	Rabbits	cornea	topical	+	Gauri and Malorny (1967)
Uridine, 2′-deoxy-5-tri-fluoromethyl-	Vaccinia (DNA)	Rabbits	cornea	topical	+	Kaufman and Heidelberger (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman and Heidelberger (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman (1965b)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hyndiuk and Kaufman (1967)
	Herpes (DNA)	Rabbits	cornea	topical	−	Hall *et al.* (1968)

TABLE 33. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF OTHER 5-SUBSTITUTED URACILS AND THEIR NUCLEOSIDES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Uracil, 5-amino-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
Uracil, 5-amino-6-methyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Dreisbach *et al.* (1949)
	Ectromelia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
Uracil, 5-amino-6-phenyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
Uracil, 5-amino-6-propyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
Uracil, 5-[bis(2-chloroethyl)-amino]-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	−	Kaufman *et al.* (1962c)
	Influenza A (RNA)	Mice	i.n.	i.p.	+	Sidwell *et al.* (1968a)
Uracil, 5,6-diamino-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
Uracil, 5-diazo-, hydrate	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Uracil, 5-hydroxy-6-methyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Dreisbach *et al.* (1949)
Uracil, 5-formamido-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Dreisbach *et al.* (1949)
Uracil, 5-nitro-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
Uracil, 5-nitro-6-methyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
5-Uracilcarboxaldehyde thiosemicarbazone	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (DNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
6-Uracilcarboxylic acid, 5-amino-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)

TABLE 33 (*cont.*)

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
6-Uracilcarboxylic acid, 5-nitro-	Vaccinia (DNA)	Mice	i.c.	p.o.	–	Dreisbach *et al.* (1949)
Uridine, 2′-deoxy-5-methylamino-	Herpes (DNA)	Rabbits	cornea	topical	+	Nemes and Hilleman (1965)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hyndiuk and Kaufman (1967)

TABLE 34. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF N-ALKYL DERIVATIVES OF URACIL

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Uracil, 1-allyl-6-chloro-3,5-diethyl-	Herpes (DNA)	Rabbits	cornea	topical	+	Gauri and Rohde (1969)
Uracil, 5-(2-chloroacetamido)-1,3-dimethyl-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Uracil, 3-(mercaptomethyl)-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	+	Sidwell *et al.* (1968a)
Uracil, 5-nitro-1,3,6-trimethyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)

TABLE 35. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF CYTOSINE DERIVATIVES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Adenosine, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Adenosine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Adenosine, 2′-deoxy-, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	–	Renis *et al.* (1967)
Aracytidine, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1967)
Aracytidine, uridylyl-[2′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Aracytidine, uridylyl-[3′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Cytidine, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	–	Renis *et al.* (1967)
Cytidine, 2′-deoxy-, aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	–	Renis *et al.* (1967)
Cytidine, 2′-deoxy-3′,5′-*O*-diacetyl-5-iodo-	Herpes (DNA)	Rabbits	cornea	topical	+	Perkins *et al.* (1962)
Cytidine, 2′-C-methyl-	Vaccinia (DNA)	Mice	i.v.	i.p.	–	Walton *et al.* (1969)
Cytidine, 3′-C-methyl-	Vaccinia (DNA)	Mice	i.v.	i.p.	+	Walton *et al.* (1969)
Cytosine, 5-amino-	Vaccinia (DNA)	Mice	i.c.	p.o.	–	Dreisbach *et al.* (1949)
Cytosine, 5-(p-aminophenyl)-	Hypr (Group B—Tickborne arbovirus) (RNA)	Mice	s.c.	s.c.	–	Libikova (1968)
Cytosine, 1-β-D-arabinofuranosyl- (ara-C)	Vaccinia (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Vaccinia (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)

TABLE 35 (*cont.*)

Compound	Virus Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Cytosine, 1-β-D-arabinofuranosyl- (ara-C) (continued)	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1962)
	Herpes (DNA)	Monkeys	cornea	topical	+	Kaufman and Maloney (1963b)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman and Maloney (1963b)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Kaufman (1965b)
	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
	Herpes (DNA)	Hamsters	cornea	topical	+	Sidwell *et al.* (1967)
	Herpes (DNA)	Rabbits	cornea	topical	+	Hall *et al.* (1968)
	Herpes (DNA)	Rabbits	cornea	topical	+	Prince *et al.* (1969)
	Herpes (DNA)	Rabbits	intra-ocular	topical	+	Kaufman *et al.* (1970)
	Coxsackie B1 (RNA)	Mice	i.p.	i.p.	−	Prince *et al.* (1969)
	Encephalomyocarditis (Columbia SK) (RNA)	Mice	i.p.	i.p.	−	Prince *et al.* (1969)
	Influenza (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza (RNA)	Mice	i.n.	i.p.	−	Prince *et al.* (1969)
Cytosine, 1-β-D-arabinofuranosyl-5-bromo-	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-5-chloro-	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1968)

TABLE 35 (*cont.*)

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Cytosine, 1-β-D-arabinofuranosyl-5-fluoro-	Herpes (DNA)	Rabbits	i.p.	i.p.	+	Prince *et al.* (1969)
	Coxsackie B1 (RNA)	Mice	i.p.	i.p.	−	Prince *et al.* (1969)
	Encephalomyocarditis (Columbia SK) (RNA)	Mice	i.p.	i.p.	−	Prince *et al.* (1969)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Prince *et al.* (1969)
Cytosine, 1-β-D-arabinofuranosyl-5-iodo-	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-3-methyl-, methylhydrogen sulfate	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1968)
Cytosine, 1-β-D-arabinofuranosyl-, 3′-phosphate	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Cytosine, 1-β-D-arabinofuranosyl-, 5′-phosphate	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood (1964)
	Herpes (DNA)	Rabbits	cornea	topical	+	Underwood *et al.* (1964)
	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Cytosine, 2′-deoxy-5-iodo-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	+	Perkins *et al.* (1962)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
Pyrimidine, 2,4-diamino-5-(p-chlorophenyl)-6-ethyl- (Daraprim)	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)

TABLE 35 (*cont.*)

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_z	Activity	Reference
Thymidine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1967)
Uridine, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Uridine, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Uridine, 2′-deoxy-, aracytidylyl-[2′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
Uridine, 2′-deoxy-, aracytidylyl-[3′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	+	Renis *et al.* (1967)
	Herpes (DNA)	Rabbits	cornea	topical	+	Renis *et al.* (1967)
Uridine, 2′-deoxy-aracytidylyl-[5′ → 5′]-	Herpes (DNA)	Mice	i.c.	i.c.	−	Renis *et al.* (1967)

TABLE 36. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 2-THIOPYRIMIDINES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Uracil, 5-(4-bromophenoxyl)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Thompson *et al.* (1951)
	Semliki Forest (RNA)	Mice	i.p.	p.o.	+	Thompson and Lavender (1953)
Uracil, 5-(4-*t*-butyl-2-chlorophenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(4-*t*-butylphenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(4-chloro-3-methyl-phenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(4-chlorophenoxy)-6-methyl-2-thio-	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Thompson *et al.* (1951)
Uracil, 5-(4-chlorophenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(2,4-dibromo-phenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(2,4-dichloro-phenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Minton *et al.* (1953)
	Vaccinia (DNA)	Mice	i.c.	s.c.	+	Bauer (1955)
	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	+	Sidwell *et al.* (1968a)

TABLE 36 (*cont.*)

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Uracil, 5-(2,4-dichlorophenoxy)-2-thio- (continued)	Semliki Forest (RNA)	Mice	i.p.	p.o.	+	Thompson and Lavender (1953)
Uracil, 5-(3,4-dichlorophenoxy)-2-thio-	Semliki Forest (RNA)	Mice	i.p.	p.o.	+	Thompson and Lavender (1953)
Uracil, 5-(3,4-dimethoxyphenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Thompson *et al.* (1951)
Uracil, 5-(3,4-dimethylphenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Thompson *et al.* (1951)
	Semliki Forest (RNA)	Mice	i.p.	p.o.	+	Thompson and Lavender (1953)
Uracil, 5-(4-ethoxyphenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Thompson *et al.* (1951)
Uracil, 5-(4-iodophenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o.	+	Thompson *et al.* (1951)
Uracil, 5-(4-methoxyphenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(4-methylphenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Uracil, 5-phenoxy-2-thio-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Uracil, 5-(2,3,4-trichlorophenoxy)-2-thio-	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Thompson *et al.* (1951)

TABLE 37. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF ISOCYTOSINE AND ALKYL DERIVATIVES

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Isocytosine	Vaccinia (DNA)	Mice	i.c.	p.o.	–	Dreisbach *et al.* (1949)
Isocytosine, 5-formamido-	Vaccinia (DNA)	Mice	i.c.	p.o.	–	Dreisbach *et al.* (1949)
Isocytosine, 5-methyl-	Vaccinia (DNA)	Mice	i.c.	p.o.	–	Dreisbach *et al.* (1949)
Pyrimidine, 2-amino-4-methoxy-	Vaccinia (DNA)	Mice	i.c.	p.o.	–	Dreisbach *et al.* (1949)

TABLE 38. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF 6-AZAPYRIMIDINES

Compound	Virus Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
6-Azacytidine	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)
6-Azauracil	Vaccinia (DNA)	Mice	i.c.	i.p.	+	Sidwell *et al.* (1968a)
	Influenza A (RNA)	Mice	i.n.	i.p.	+	Sidwell *et al.* (1968a)
6-Azauridine	Vaccinia (DNA)	Mice	i.n.	s.c.	+	Jasinska *et al.* (1962)
	Vaccinia (DNA)	Mice	i.c.	s.c.	−	Jasinska *et al.* (1962)
	Vaccinia (DNA)	Mice	i.c.	i.p.	−	Sidwell *et al.* (1968a)
	Herpes (DNA)	Rabbits	cornea	topical	+	Galegov *et al.* (1970)
	Influenza A (RNA)	Mice	i.n.	i.p.	−	Sidwell *et al.* (1968a)

TABLE 39. REPORTED TESTING FOR *IN VIVO* ANTIVIRAL ACTIVITY OF MISCELLANEOUS PYRIMIDINES

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Barbituric acid, sodium salt (Barbiturate)	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
Barbituric acid, 3-allyl-,	Japanese encephalitis (RNA)	Mice	—	i.v.	+	Mizuma *et al.* (1961)
Barbituric acid, 5-allyl-5-(2-cyclopenten-1-yl)- (Cyclopal)	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
Barbituric acid, 5-allyl-5-pentadecyl-2-thio-	Influenza A1, A2, B (RNA)	Mice	i.n.	p.o., i.p., i.v.	+	Mosimann (1966)
	Influenza A (PR8) (RNA)	Mice	i.n.	p.o., i.p., i.v.	−	Mosimann (1966)
	Parainfluenza 1 (Sendai) (RNA)	Mice	i.n.	p.o., i.p., i.v.	+	Mosimann (1966)
Barbituric acid, 3-benzyl-5,5-diethyl-	Japanese encephalitis (RNA)	Mice	—	i.v.	+	Mizuma *et al.* (1961)
Barbituric acid, 3-benzyl-5-ethyl-5-phenyl-	Japanese encephalitis (RNA)	Mice	—	i.v.	+	Mizuma *et al.* (1961)

TABLE 39 (*cont.*)

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Barbituric acid, 3-benzyl-5-methyl-	Japanese encephalitis (RNA)	Mice	—	i.v.	+	Mizuma *et al.* (1961)
Barbituric acid, 3-butyl-5-methyl-5-lauryl-	Japanese encephalitis (RNA)	Mice	—	i.v.	+	Mizuma *et al.* (1961)
Barbituric acid, 5,5-diethyl-, sodium salt (Barbital)	Polio (RNA)	Mice	i.c.	i.p.	–	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	–	Kramer *et al.* (1944)
Barbituric acid, 5-ethyl-, sodium salt (Neonal)	Polio (RNA)	Mice	i.c.	i.p.	–	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	–	Kramer *et al.* (1944)
Barbituric acid, 5-ethyl-5-butyl-, sodium salt	Polio (RNA)	Mice	i.c.	i.p.	–	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	–	Kramer *et al.* (1944)
Barbituric acid, 5-ethyl-5-(3′-4′-dichlorophenyl)-	Coxsackie A21 (RNA)	Mice	—	oral, i.p.	+	DeLong *et al.* (1970)
Barbituric acid, 5-ethyl-5-hexyl-, sodium salt (Ortal)	Polio (RNA)	Mice	i.c.	i.p.	–	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	–	Kramer *et al.* (1944)

TABLE 39 (*cont.*)

Compound	Virus: Name (Type)	Host	Route of virus inoc.	Route of R_x	Activity	Reference
Barbituric acid, 5-ethyl-5-(1-methylbutyl)-2-thio-, sodium salt	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
Barbituric acid, 5-ethyl-5-isoamyl-, sodium salt (Amytal)	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
Barbituric acid, 5-ethyl-5-(1-methylbutyl)-, sodium salt (Pentobarbital)	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
Barbituric acid, 5-ethyl-5-phenyl-, sodium salt (Phenobarbital)	Polio (RNA)	Mice	i.c.	i.p.	−	Kramer *et al.* (1944)
	St. Louis encephalitis (RNA)	Mice	i.n.	i.p.	−	Kramer *et al.* (1944)
Barbituric acid, 3-methyl-5-propyl-	Japanese encephalitis (RNA)	Mice	—	i.v.	+	Mizuma *et al.* (1961)
Pyrimidine, 2-amino-4-mercapto-5-(2′chlorophenoxy)-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)

TABLE 39 (*cont.*)

Compound	Virus	Host	Route of virus inoc.	Route of R_x	Activity	Reference
	Name (Type)					
Pyrimidine, 2-amino-4-mercapto-5-(4′-chlorophenoxy)-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Pyrimidine, 2-amino-4-mercapto-5-phenoxy-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Pyrimidine, 2,4-dimercapto-5-(2′,4′-dichlorophenoxy)-	Vaccinia (DNA)	Mice	i.c.	p.o., i.p.	+	Thompson *et al.* (1951)
Uracil, 6-amino-	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)
Uramil	Vaccinia (DNA)	Mice	i.c.	p.o.	−	Dreisbach *et al.* (1949)

TABLE 40. CLINICAL REPORTS OF THE ANTIVIRAL ACTIVITY OF PYRIMIDINES IN VIRUS INFECTIONS IN MAN

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
5-Azaorotic acid	Vaccinia (DNA)	Primary vaccination lesion	i.v.	−	Calabresi (1965)
6-Azuridine	Vaccinia (DNA)	Primary vaccination lesion	i.v.	−	Calabresi (1965)
	Variola (DNA)	Smallpox	i.v.	+	Jaffari and Hussain (1969)
	Herpes (DNA)	Keratitis	oral	+	Myska *et al.* (1967)
	Varicella-zoster (DNA)	Keratitis	oral	+	Myska *et al.* (1967)
Cytidine, 2′-deoxy-5-iodo-	Vaccinia (DNA)	Primary vaccination lesion	i.v.	+, −	Calabresi (1965)
Cytosine, 1-β-D-arabino-furanosyl- (ara-C)	Vaccinia (DNA)	Primary vaccination lesions	i.v.	−	Calabresi (1965)
	Herpes (DNA)	Keratitis	topical	+	Kaufman and Maloney (1963b)
	Herpes (DNA)	Pneumonia	i.v.	−	Douglas *et al.* (1969)
	Herpes (DNA)	Severe encephalitis	intra-thecal, i.v.	+	Chow *et al.* (1970)
	Herpes (DNA)	Disseminated infections; oral, pharyn-geal and lung lesions	i.v.	+	Juel-Jensen (1970c)

TABLE 40 (*cont.*)

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
Cytosine, 1-β-D-arabino-furanosyl- (ara-C) (continued)	Herpes (DNA)	Disseminated infection	i.v.	+	Juel-Jensen (1970d)
	Varicella-zoster (DNA)	Disseminated varicella	i.v.	+	Calabresi (1965)
	Varicella-zoster (DNA)	Disseminated varicella	i.v.	+	Hall *et al.* (1968); Hall *et al.* (1969); Kwaan *et al.* (1969)
	Varicella-zoster (DNA)	Disseminated varicella	i.v.	+	McKelvey and Kwaan (1969)
	Varicella-zoster (DNA)	Zoster	i.v.	−	Wiernik and Serpick (1969)
	Varicella-zoster (DNA)	Disseminated zoster	i.v.	+	Chow *et al.* (1970)
	Varicella-zoster (DNA)	Zoster	i.v.	+	Chow *et al.* (1970)
	Varicella-zoster (DNA)	Disseminated varicella	s.c.	−	Seligman and Rosner (1970)
	Varicella-zoster (DNA)	Disseminated varicella	i.v.	+	Prager *et al.* (1971)
Uracil, 1-allyl-6-chloro-3,5-diethyl-	Herpes (DNA)	Cutaneous lesions, stomatitis, herpes genitalis	topical	+	Gauri and Rohde (1969)
Uracil, 5-fluoro-	Papilloma (DNA)	Warts	topical	+	Hursthouse (1970)
Uracil, 5-nitro-	Varicella-zoster (DNA)	Zoster	topical	+	Murthy and Testa (1966)

TABLE 40 (*cont.*)

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
Uridine, 5-bromo-2′-deoxy-	Measles (RNA)	Subacute sclerosing panencephalitis (SSPE) or Dawson's inclusion body encephalitis (DIBE)	i.v.	+	Freeman (1968); Freeman (1969); Kertesz *et al.* (1970)
Uridine, 5-chloro-2′-deoxy-	Herpes (DNA)	Keratitis	topical	+	Gordon and Karnofsky (1962)
Uridine, 2′-deoxy-5-ethyl-	Herpes (DNA)	Keratitis	topical	+	De Decker (1969)
	Herpes (DNA)	Keratitis	topical	+	Riehm and Gauri (1969)
Uridine, 2′-deoxy-5-fluoro- (FUDR)	Vaccinia (DNA)	Primary vaccination lesion	i.v.	−	Calabresi (1965)
	Cytomegalo (DNA)	Pneumonitis	i.v.	+	Cangir *et al.* (1967)
Uridine, 2′-deoxy-5-iodo- (IDU)	Vaccinia (DNA)	Primary vaccination lesion	i.v.	+	Calabresi *et al.* (1962); Calabresi (1963)
	Vaccinia (DNA)	Primary vaccination lesion	i.v.	+	Calabresi *et al.* (1963)
	Vaccinia (DNA)	Primary vaccination lesion	topical	−	Bjornberg *et al.* (1964)

TABLE 40 (*cont.*)

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Vaccinia (DNA)	Primary vaccination lesion	i.v.	+	Calabresi (1965)
	Vaccinia (DNA)	Vaccinia gangrenosa	i.v.	+, −	Calabresi (1965)
	Vaccinia (DNA)	Vaccinia gangrenosa	topical i.v.	−	Boughton *et al.* (1969)
	Vaccinia (DNA)	Keratitis	topical	+	Vitiello (1967)
	Vaccinia (DNA)	Keratitis	topical	+	Jelinek (1969)
	Herpes (DNA)	Keratitis	topical	+	Bennett (1962)
	Herpes (DNA)	Keratitis	topical	+	Corrigan *et al.* (1962)
	Herpes (DNA)	Keratitis	topical	+	Davidson (1962)
	Herpes (DNA)	Keratitis	topical	+	Gordon and Karnofsky (1962)
	Herpes (DNA)	Keratitis	topical	+	Kaufman *et al.* (1962d)
	Herpes (DNA)	Keratitis	topical	+	Kaufman *et al.* (1962a)
	Herpes (DNA)	Keratitis	topical	+	Thiel and Wacker (1962)
	Herpes (DNA)	Acute keratitis	topical	+	Burns (1963)
	Herpes (DNA)	Chronic recurrent keratitis	topical	−	Burns (1963)
	Herpes (DNA)	Keratitis	topical	+	Gilkes (1963)
	Herpes (DNA)	Keratitis	topical	+	Havener and Wachtel (1963)

TABLE 40 (*cont.*)

Compound	Virus	Clinical diagnosis	Route of treatment	Activity	Reference
	Name (Type)				
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Herpes (DNA)	Keratitis	topical	+	Hertzberg (1963)
	Herpes (DNA)	Keratitis	topical	+	Maxwell (1963)
	Herpes (DNA)	Keratitis	topical	+	Patterson *et al.* (1963)
	Herpes (DNA)	Keratitis	topical	+	Thygeson *et al.* (1963)
	Herpes (DNA)	Keratitis	topical	+	Davidson and Evans (1964)
	Herpes (DNA)	Keratitis	topical	+	Jepson (1964)
	Herpes (DNA)	Keratitis	topical	+	Laibson and Leopold (1964)
	Herpes (DNA)	Keratitis	topical	+	Gold *et al.* (1965)
	Herpes (DNA)	Keratitis	topical	+	Hart *et al.* (1965)
	Herpes (DNA)	Keratitis	topical	+	Patterson and Jones (1967)
	Herpes (DNA)	Keratitis	topical	+	Graupner and Müller (1968)
	Herpes (DNA)	Keratitis	topical	−	Graupner *et al.* (1969)
	Herpes (DNA)	Keratitis	topical	+	Morgan (1970)
	Herpes (DNA)	Keratitis	topical[1]	+	Gordon and Karnofsky (1962)
	Herpes (DNA)	Keratitis	topical[2]	+	Kaufman *et al.* (1963)
	Herpes (DNA)	Conjunctival ulceration	topical	−	Nauheim (1969)
	Herpes (DNA)	Cutaneous lesions	topical	+	Hall-Smith *et al.* (1962)

[1]IDU + FUDR
[2]IDU + Corticosteroids

TABLE 40 (*cont.*)

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Herpes (DNA)	Cutaneous lesions	topical	−	Burnett and Katz (1963)
	Herpes (DNA)	Cutaneous lesions	topical	+	Jackson (1963)
	Herpes (DNA)	Cutaneous lesions	topical	+	Tomkins (1963)
	Herpes (DNA)	Recurrent cutaneous lesions	topical	−	Ive (1964)
	Herpes (DNA)	Cutaneous lesions	topical	−	Juel-Jensen and MacCallum (1964)
	Herpes (DNA)	Cutaneous lesions	i.d.	+	Juel-Jensen and MacCallum (1965)
	Herpes (DNA)	Cutaneous lesions	topical	+	Gold *et al.* (1965)
	Herpes (DNA)	Cutaneous lesions	topical	+	Goldman and Kitzmiller (1965)
	Herpes (DNA)	Cutaneous lesions	topical	+	Corbett *et al.* (1966)
	Herpes (DNA)	Cutaneous lesions	topical	+	MacCallum and Juel-Jensen (1966)
	Herpes (DNA)	Vulvitis	topical	+	Dickie (1969)
	Herpes (DNA)	Herpes labialis	topical	−(?)	Gardner and Tennant (1969)

TABLE 40 (*cont.*)

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Herpes (DNA)	Oral infections	topical	+	Najjar *et al.* (1969)
	Herpes (DNA)	Cutaneous lesions	topical	+	Turnbull *et al.* (1969)
	Herpes (DNA)	Herpetic whitlow	topical	+	Juel-Jensen (1970a)
	Herpes (DNA)	Cutaneous lesions	topical	−	Kibrick and Katz (1970)
	Herpes (DNA)	Cutaneous lesions	topical	+	Longson (1970)
	Herpes (DNA)	Herpes genitalis	topical	+	Hutfield (1964)
	Herpes (DNA)	Herpes progenitalis	topical	+	Schofield (1964)
	Herpes (DNA)	Herpes labialis	topical	+	Bánóczy (1967)
	Herpes (DNA)	Acute stomatitis and pharyngitis	topical	+	Alexander (1969)
	Herpes (DNA)	Congenital disseminated infection	topical, i.v., i.m.	+ +	Partridge and Millis (1968)
	Herpes (DNA)	Kaposi's varicelliform eruption	topical	+	Castrow and Chernosky (1969)

TABLE 40 (*cont.*)

Compound	Virus: Name (Type)	Clinical diagnosis	Route of treatment	Activity	Reference
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Herpes (DNA)	Encephalitis	i.v.	+	Breeden *et al.* (1966)
	Herpes (DNA)	Encephalitis	intra-carotid	+	Buckley and MacCallum (1967)
	Herpes (DNA)	Encephalitis	i.v.	+	Evans *et al.* (1967)
	Herpes (DNA)	Encephalitis	i.v.	+	Marshall (1967)
	Herpes (DNA)	Encephalitis	i.v.	+	Page *et al.* (1967)
	Herpes (DNA)	Encephalitis	i.v.	+	Bellanti *et al.* (1968)
	Herpes (DNA)	Encephalitis and disseminated infection	i.v.	+	Golden *et al.* (1969)
	Herpes (DNA)	Encephalitis	i.v.	+	Nolan *et al.* (1969)
	Herpes (DNA)	Encephalitis	i.v.	−	Dayan & Lewis (1969)
	Herpes (DNA)	Encephalitis	i.v.	+	Rappel and Brihaye (1969)
	Herpes (DNA)	Encephalitis	i.v.	+	Tuffli and Nahmias (1969)
	Herpes (DNA)	Encephalitis	i.v.	+	Charnock and Cramblett (1970)
	Herpes (DNA)	Encephalitis	i.v.	+	Goldman *et al.* (1970)
	Herpes (DNA)	Encephalitis	not given	+	Lerner *et al.* (1970)
	Herpes (DNA)	Encephalitis	i.v.	+	Meyer *et al.* (1970)
	Herpes (DNA)	Encephalitis	not given	+	Overgaard *et al.* (1971)
	Herpes (DNA)	Encephalitis	i.v.	+	Silk and Roome (1970)

TABLE 40 (*cont.*)

Compound	Virus	Clinical diagnosis	Route of treatment	Activity	Reference
	Name (Type)				
Uridine, 2′-deoxy-5-iodo- (IDU) (continued)	Herpes (DNA)	Encephalitis	not given	+	Tomlinson and MacCallum (1970)
	Herpes (DNA)	Encephalitis	i.v. and intra-thecal	+	Wenzl and Rubio (1970)
	Varicella-zoster (DNA)	Zoster	topical	−	McCallum *et al.* (1964)
	Varicella-zoster (DNA)	Varicella	i.v.	+	Calabresi (1965)
	Varicella-zoster (DNA)	Disseminated zoster	not given	+	Waltuch and Sachs (1968)
	Varicella-zoster (DNA)	Zoster	topical	+	Juel-Jensen (1970b)
	Cytomegalo (DNA)	Congenital cytomegalovirus infection	i.v.	+	Conchie *et al.* (1968)
	Adeno (DNA)	Conjunctivitis	topical	−	Dudgeon *et al.* (1969)
Uridine, 5-trifluoromethyl-	Adeno 3 (DNA)	Conjunctivitis and pre-auricular adenopathy	topical and oral	−	Little *et al.* (1968)

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CHAPTER 5

VIRUS-INDUCED INTERFERONS

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INTRODUCTION

INTERFERONS are a group of antiviral proteins produced by cells in response to virus infection or exposure to some nonviral substances. Treatment with an interferon confers upon cells resistance to multiplication of a variety of viruses. This review concerns interferon induced by virus infection.

Interferon was discovered by Isaacs and Lindenmann (1957) during investigation of interference by influenza virus; however, several workers in previous studies had made observations which subsequent to the discovery of Isaacs and Lindenmann could be best explained by invoking an interferon mechanism (Orskov and Anderson, 1938; Gard, 1944; Lennette and Koprowski, 1946). (Interference is the ability of one virus, active or inactivated, to suppress the replication of another related or unrelated virus.) The contribution of Isaacs and Lindenmann was to show that in some, but not all, cases, interference was due to a humoral substance (later shown to be a protein) released into body fluids or tissue culture medium. Incubation of cells with fluids containing this protein conferred resistance to virus infection upon these cells. The discovery of interferon stimulated a great deal of interest since a wide variety of viruses caused the production of interferons and the growth of an equally wide variety of viruses could be suppressed by interferon treatment.

The discovery of interferon was also of very great theoretical interest and possibly of practical value since it helped to explain some cases of interference (Friedman, 1964). There was also strong evidence that interferon was involved in the mechanism for natural recovery from virus

infections (Friedman *et al.*, 1962; Baron and Buckler, 1963). Lastly, the discovery of interferon gave rise to the first reasonable hope that the development of a general prophylactic agent for virus infections and substances of use in treating established virus infections would be possible.

For these reasons there has been a rapid accumulation of findings on interferon, and because of this large volume of data the general plan for this presentation will be to concentrate on interferon studies in a few typical virus-cell systems which are fairly well understood. Other significant contributions to studies on interferon will be mentioned where these will add significantly to the reader's understanding of the mechanisms of action and production, and the biological significance of interferons. The presentation will follow the above mentioned plan, and will not attempt a chronological or complete cataloguing of interferon studies.

The review has the following general outline. The properties of interferons and various systems for their assay will be listed together with a very brief survey of purification procedures. A discussion of the replication mechanism for an RNA virus, Semliki Forest virus (arbovirus group A), and a DNA virus, vaccinia virus, follows. These viruses were chosen because many studies on the mechanism of action and production of interferons, detailed in the two following sections, have been performed with these viruses. The next sections concern interferons as natural inhibitors of virus infections and possible therapeutic and prophylactic uses of interferons. The last section deals with their biological significance.

PROPERTIES OF INTERFERONS

(a) BIOLOGICAL PROPERTIES

The most significant property of interferons is obviously their ability to inhibit virus replication. This is apparently accomplished with minimal alteration in the physiological processes of the cell (Levy and Merigan, 1966). Interferons are probably related to a normal mechanism of cell resistance to virus infection since their antiviral activity is remarkably broad in range, generally most effective against arboviruses and some picornaviruses but also quite effective against myxoviruses and reoviruses, other important animal RNA virus groups (Isaacs, 1963). Interferon clearly inhibits the growth of many RNA tumor viruses, but sufficient information on the relative sensitivity of this group is not yet available, so that it is not possible to compare its inhibitory action on RNA tumor viruses with its action on other RNA virus groups (Bader, 1962; Standström *et al.*, 1962).

Interferon also inhibits the growth of DNA viruses. It is fairly effective against vaccinia virus and other poxviruses, agents which grow in the cytoplasm (Isaacs, 1963). Interferon also inhibits the replication of the papova viruses (Oxman and Black, 1966), and is effective against some strains of herpes simplex virus, all of which replicate in the nucleus (Glasgow and Habel, 1962); however, adenoviruses, which also replicate in the nucleus, are quite resistant to interferon (Cantell, 1961). This may prove of importance in the analysis of interferon action, since it would be of interest to discover why adenoviruses are so resistant. This wide spectrum of antiviral activity is a puzzling property. It suggests some common virus structure or site of activity in the cell for many diverse animal virus groups; however, any comprehensive theory seeking to explain interferon action would have to account for both the marked resistance of some groups of viruses to interferon and the differing sensitivities of some strains of the same virus.

Another biological property of interferon of great interest, is its species specificity (Tyrrell, 1959). The term species specificity has been criticized as being inaccurate (Bucknall, 1967) and genus specificity would be more correct, but it is so well accepted that it will be employed in this discussion. Species specificity refers to the observation that the antiviral activity of an interferon preparation is most pronounced in the tissues of the animal in which the preparation was made and the activity decreases when the preparation is employed in the tissues of dissimilar animals. For instance, a mouse interferon preparation which is very effective in mice or in most cultures of mouse cells will be slightly effective in the cells of very closely related rodents such as hamsters (Buckler and Baron, 1966), but completely ineffective in more distantly related animals such as rabbits or chickens. There is some cross between primate interferons (Isaacs *et al.*, 1961) but human interferon is not effective in lower animals (Merigan *et al.*, 1965). As might be expected, an exception to this specificity has been found: a human interferon is effective in rabbit cells (Desmyter *et al.*, 1968). Otherwise, the specificity rule holds up quite well, especially when considering the activity of purified interferon preparations (Merigan, 1964), and reports of heterologous activity of interferons must therefore be carefully considered before they can be accepted (Buckler and Baron, 1966).

The remarkable specificity of interferon action was perhaps the first indication that its mechanism of action might in some way be related to the genetic apparatus of the cell. A basis for such exact specificity could lie in the complex structure of DNA. Cross-reactivities in closely related species may very well be due to the existence of similar nucleotide sequences

which carry out the same function related to interferon action. As animals diverge in an evolutionary sense the structure of these sequences would be expected to change and, therefore, be very unlikely to respond to the interferon of distantly related animals.

Even among cell lines from the same species, however, sensitivity to interferon varies (Chany, 1961; Lockhart, 1965); in fact, in primary cell cultures, sensitivity to interferon changes with the age and metabolic state of the culture (Ho and Enders, 1959). Again, in attempting to explain interferon action it would be important to account for these variations.

Interferons are antigenically distinct from viruses; therefore, antiviral antibody does not neutralize interferon (Isaacs *et al.*, 1957). It has been difficult to produce effective antisera against interferons (Paucker and Cantell, 1962; Paucker, 1965) but the reason for this now seems evident. As explained below, the specific activity of interferon on a weight basis is extremely high so that injection into animals of even large amounts of biologically active interferon appears to present them with a very small antigenic mass (Fantes, 1966a, b). It is, therefore, not too surprising that production of potent anti-interferon antisera has been slow and difficult.

(b) CHEMICAL AND PHYSICAL PROPERTIES

In discussing the chemical and physical properties of interferons two general observations are pertinent. Firstly, all of these properties are assayed, finally, in a biological system based on virus growth inhibition. Lack of an exact chemical or physical test for interferon has made a precise definition of some of its properties difficult. Secondly, it has been impossible to purify interferons completely. The properties of partially purified and crude interferons differ and many of the chemical and physical properties were originally determined on crude interferon, so that much of the material concerning these in early reports on interferons is incorrect (Fantes, 1966a, b). Since absolutely pure interferons are not yet available, it is not possible to determine many of their potentially important properties. In general, the discussion will be limited to the properties of partially purified interferons.

Despite the differences which must exist between interferons of different animals and the various interferons found in the same animal, it is possible to generalize about many of the properties of interferons. The molecular weight of various virus-induced interferons as determined by gel filtration and sedimentation falls in the 20,000 to 40,000 Dalton range (Fantes, 1966a, b) but interferons induced in animals by nonviral inducers often

have molecular weights greater than 50,000 (Hallum *et al.*, 1965). In a given animal tissue culture, however, different viruses induce interferon preparations with similar properties (Fantes, 1966a, b). Partially purified chick interferon preparations induced by either influenza or herpes virus have identical properties (Lampson *et al.*, 1965).

The sensitivity of all interferons to hydrolysis by proteolytic enzymes and their insensitivity to nucleases, sialidases, lipases, etc., indicate that interferons are proteins (Fantes, 1966a, b). If they contain other structural elements than amino-acids, these are very minor. Interferons have isoelectric points between 6.5 and 7.5 (Fantes, 1966a, b). Disulphide groups appear necessary for antiviral activity (Fantes and O'Neil, 1964). Amino-acid analysis of partially purified preparations of interferon indicated the presence of all the usual amino-acids but this analysis also indicated the presence of glucosamine (Fantes, 1966a, b). Such studies would suggest that interferons are glycoproteins, but subsequent work showed the preparations to be less pure than was originally thought (Fantes, 1966a, b), for several lines were present when electrophoresis in polyacrylamide gels was carried out on them. Therefore, there is no doubt that interferons are proteins; they may be glycoproteins but this is unlikely. They are definitely not lipoproteins or nucleoproteins.

The antiviral activity of interferons is a remarkably stable property, for it is not destroyed over a wide pH range, from pH 1 to pH 10 in most cases. This unusual stability is quite useful in allowing the destruction of infectious activity of most viruses used to induce interferons while preserving the antiviral activity of the preparation (Lindenmann *et al.*, 1957). The antiviral activity of interferons is also moderately stable to heat, but here species differences exist. Mouse (Lampson *et al.*, 1966) and chick (Lampson *et al.*, 1965) interferons are stable at temperatures up to 65° but human interferon is destroyed at 56° (Merigan *et al.*, 1966). In general, all interferons are stable to exposure to moderately high temperatures for long periods but are rapidly destroyed by boiling (Fantes, 1966a, b). Partially purified but not crude interferon preparations are inactivated by ultraviolet light (Lampson *et al.*, 1963) and by several cycles of freezing and thawing (Merigan *et al.*, 1965).

(c) CRITERIA FOR ESTABLISHING AN ANTIVIRAL SUBSTANCE AS AN INTERFERON

Because of the inability so far to obtain completely purified interferon preparations, and also because of the variations in the properties of

interferons from different species (and even interferons from the same species) it is hard to be dogmatic about what exactly constitutes an interferon. Several properties seem reasonably constant, however, among interferons so far studied, and any antiviral substance considered to be a virus-induced interferon should have the following properties. It should be a protein, the antiviral activity of which is stable to a broad pH range and to at least 56° for 30 min; human interferon is an exception to this rule since it is partially inactivated at 56°. The preparation should be active against groups of viruses which are usually considered sensitive to interferons, be more active in the cells of the species in which it was induced than in the cells of even closely related animal species and be completely inactive in distantly related animal species. Exceptions to the latter rule are possible but must be carefully studied. As will be discussed below, it is also important to ascertain whether production and action of the putative interferon are inhibited by treatment of cells with actinomycin D. In all known cases a dose of this antibiotic sufficient to inhibit cell RNA synthesis also inhibits the action of interferon and the production of virus-induced interferon.

Any substance thought to be a virus-induced interferon should possess the above discussed properties. Other determinations such as molecular weight and isoelectric point are useful and should be made where possible.

ASSAY OF INTERFERON

Basically, interferon assays are simply biological tests of ability to inhibit virus replication under standard conditions. The various assay systems employed involve, therefore, all the inaccuracies and difficulties inherent in a biological assay system. Unfortunately, no useful alternative assay systems have as yet been found.

The interferon titer of a preparation is usually indicated in units of antiviral activity. However, to date no international standards have been adopted, so that a "unit" is defined differently in each laboratory carrying out work on interferon. Most often a unit is the reciprocal of the dilution of interferon which inhibits by 50% the growth or plaque formation by a specific virus in a defined tissue culture system (Isaacs, 1963). Many laboratories demand higher levels of antiviral activity as an indication of definite interferon action (Baron *et al.*, 1967). It would certainly be of great benefit to scientists studying interferon to adopt specific standards for the interferons most commonly employed in their work—at least for chick, mouse, human, rat and rabbit interferons.

The most commonly employed techniques for assaying interferon depend on inhibition of virus cytopathic effects, either by visual observation of interferon-treated and virus-infected cells or by inhibition of virus plaque production (Finter, 1966a). One popular variation of this method is based on the reduction in virus growth seen in interferon-treated cell cultures (Sreevalsan and Lockhart, 1962). The advantages and disadvantages of this method are discussed below. Finally, the original method employed by Isaacs and Lindenmann in their discovery of, and first experiments with, interferon employs a somewhat different principle. Their assay was based on the production of a virus hemagglutinin by influenza virus (Isaacs and Lindenmann, 1957). Since only part of the hemagglutinin produced in influenza infection is virus-associated, the assay in part tested for the yield of a virus product, not necessarily infectious virus.

Details on most of the various assay methods employed and an excellent evaluation of each method are provided in a review by Finter (1966a). This work should be consulted for details.

The yield assay for interferon has several distinct advantages, which will be discussed since much of the useful information which has been obtained on interferon production and action employs this method of assay. The assay is performed (Fig. 1) by treating cells with an interferon preparation, washing the cells, and then infecting them with a high multiplicity of an interferon-sensitive virus which is capable of growing to high titers in the test cell system. The virus is allowed to grow until the midpoint (Taylor, 1964) or the end (Baron *et al.*, 1967) of the log phase of virus growth is reached. The virus yield is then harvested and assayed. The log of the virus yield varies inversely with the interferon concentration (Fig. 2). The observed antiviral action is only on the virus produced in a

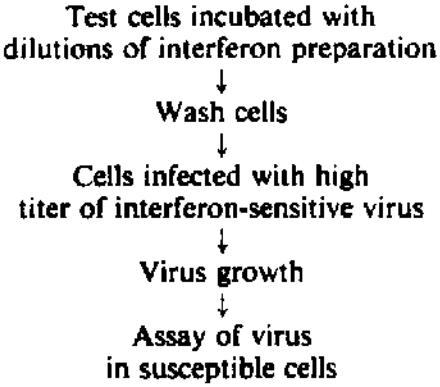

FIG. 1. Methodology of yield assay for interferon.

single growth cycle. A virus which is sensitive to interferon but a poor inducer of it can be used, but if a rapidly growing virus is employed, the endogenous production of interferon by the system does not appear to be a factor in determining the virus yield. An advantage of this assay system is that it permits fairly extensive work to be carried out on tissue cultures difficult to obtain. For instance, studies on human interferon are of obvious interest, but human tissue for cell culture is often difficult to obtain regularly. With the yield assay method, however, very small monolayers can be used for the virus growth phase of an experiment and the virus yield obtained can be assayed in another, easily obtainable cell culture system on which the test virus can grow to high titers (Friedman and Cooper, 1967).

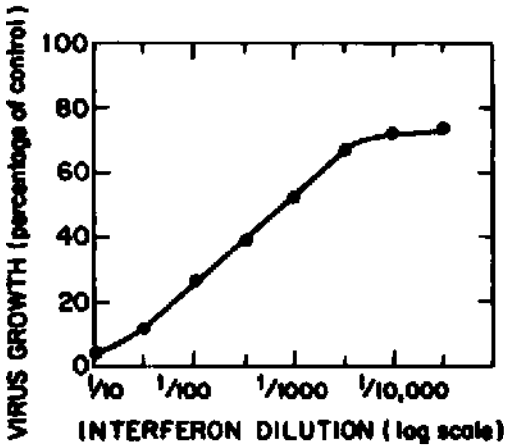

FIG. 2. Results of a virus yield assay experiment. Human interferon was titrated in a line of human skin fibroblasts (HAS I cells). The cells were challenged with Semliki Forest virus and the virus yields assayed in primary chick embryo cultures. Virus growth varies inversely with the log of the interferon dilution.

The obvious disadvantage of this assay system is that it is cumbersome and slow and demands a great deal of work, since each experimental specimen must be assayed in several dilutions to ensure accurate determination of the virus titer obtained.

Assays of interferon based on the inhibition of the synthesis of virus-specific products such as nucleic acids, enzymes or other non-structural and structural virus proteins could be developed, and might very well be simpler and faster to run than are the biological assays in current use. It appears, however, that an assay for production of infectious virus is theoretically more sensitive than such assays for virus products could be. This is because, as discussed below, interferon inhibits virus growth by

indirectly inhibiting virus protein synthesis. The production of infectious virus is the end point of a series of synthetic steps carried out under virus control. A small inhibition of virus protein synthesis might not show up significantly if only the production of a single virus protein or of virus DNA or RNA is measured. When, however, the synthesis of the final product, the infectious virus, is assayed, even minimal disturbances in virus protein synthesis may show up as large effects (Mecs *et al.*, 1967).

Other approaches to interferon assay might be fruitful. For instance, a radioimmunoassay such as is employed for polypeptide hormones (Potts *et al.*, 1967) might be as sensitive as assays based on the inhibition of virus replication. Also, an assay based on complement-fixation might make use of the remarkable specificity and sensitivity of this system (Müller-Eberhard, 1968).

PURIFICATION OF INTERFERON

For both historical and practical reasons most purification studies have been carried out on chick interferon, for this was the interferon first studied by Isaacs and Lindenmann (1957). Crude chick interferon is stable and easy to prepare in large quantities. Chick interferon has been partially purified by several methods. These have included various combinations of adsorption and elution (Fantes and O'Neil, 1964), column chromatography (Burke, 1961), gel filtration (Merigan *et al.*, 1965), precipitation of protein contaminants (Lampson *et al.*, 1963), or of interferon (Burke, 1961), and acrylamide gel electrophoresis (Merigan *et al.*, 1965). For a very complete discussion of the details of these methods and their application to the purification of chick and other interferons see the review by Fantes (1966b).

As mentioned previously, no preparation of interferon has been pure enough to carry out such important studies as a definitive amino-acid analysis. The most highly purified specimen so far obtained has been a chick interferon with a specific activity of 1.6×10^6 units per mg of protein, about a 20,000-fold purification (Fantes, 1965). When this material was analysed by electrophoresis on polyacrylamide gels at an acid pH two protein bands were found. Somewhat surprisingly, the peak of antiviral activity was found between the two stained protein peaks (Fantes, 1966a). This indicated that there was not enough interferon present ($> 10\ \mu g$) in even this preparation to form a visible band in the gel and that the true specific activity of chick interferon must be much higher than 1.6×10^6 units per mg of protein. The results suggested that one (or fewer) molecule

of chick interferon is sufficient to protect at least partially a single chick cell. This is consistent with the findings on the mechanism of action of interferon discussed below. Purified chick interferon stimulated in eggs by either influenza virus or herpes virus had the same physical and chemical properties (Lampson *et al.*, 1965).

The purification of other interferons especially from mouse (Lampson *et al.*, 1966) and human (Merigan *et al.*, 1966) sources has been attempted but with even less success than has been attained with chick interferon.

REPLICATION MECHANISMS OF ANIMAL VIRUSES

In order for the reader to understand the sections which follow it is necessary to review briefly studies which have been carried out on the mechanism of replication of animal viruses. For this purpose attention will be focused on an RNA and a DNA virus which are both fairly good producers of interferon and sensitive to its action. The RNA virus discussed is a member of the arbovirus A group, Semliki Forest virus; the DNA virus, vaccinia virus, a poxvirus. These agents have an inner core which contains their nucleic acid and an outer membrane made up of lipid, phospholipid, and protein (Osterreith and Calberg-Bacq, 1966; Zwartouw, 1964). Both viruses replicate in specific sites or factories in the cell cytoplasm (Joklik and Becker, 1964; Grimley *et al.*, 1968). Also, structural proteins may be responsible for the inhibition of cellular functions which occurs during infection with these viruses (Moss, 1968; Lust, 1966).

The outer membrane is necessary for the infectivity of Semliki Forest virus as the core is not infectious under ordinary assay conditions even though it contains RNA which upon release from the core structure is infectious (Friedman and Berezesky, 1967) when special conditions for determining RNA infectivity are employed. This probably means that the virion membrane is necessary for virus adsorption to the cells. Once the virion is adsorbed, it enters the cell and either temporarily retains its outer membrane within cytoplasmic vesicles or enters the cytoplasm directly as a virus core which has lost its outer membrane (Grimley *et al.*, 1968). The protein coat of the core is then removed; this frees the virus RNA and permits it to function as a genetic unit (Fig. 3).

The free virus RNA is now faced with the problem of initiating synthesis of virus proteins. Exactly how this occurs is at present unknown, but it has been suggested that the virus RNA forms bind to a 40S ribosomal subunit and then are translated by forming a polysome (Levy and Carter, 1968) in

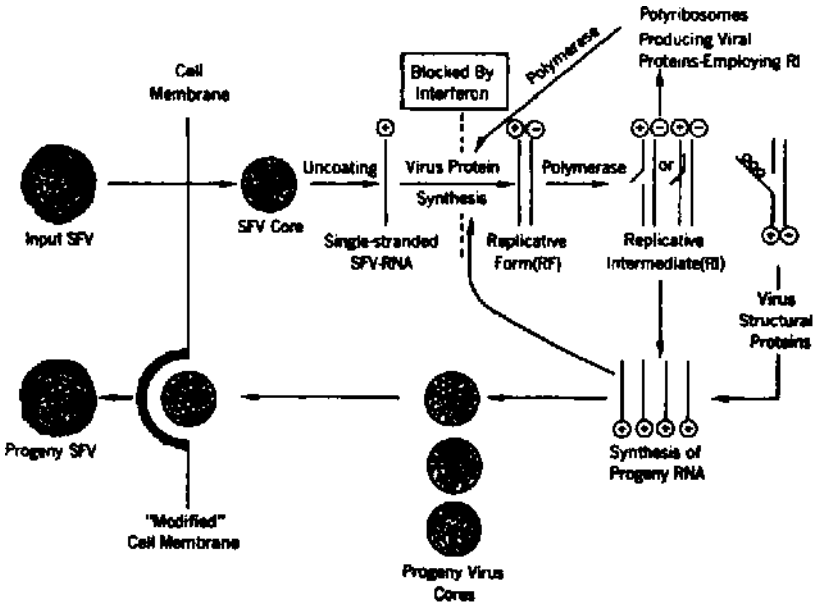

FIG. 3. Replication of Semliki Forest virus (SFV). The site of interferon action appears to be the inhibition of virus protein synthesis directed by the input (parental) virus RNA. The strands of RNA identical in base composition to the RNA of the infectious virus are labeled (+). The strands complementary to these are labeled (−).

a manner similar to that by which cell messenger RNA is thought to form polysomes (Joklik and Becker, 1965). If this is true it is quite different from the manner in which protein synthesis is carried out later in Semliki Forest virus infection (Friedman, 1968a). However, for virus replication to take place it is essential that virus proteins be made immediately after infection. The reason for this is that both virus protein and RNA are ordinarily produced on a virus RNA structure, the replicative intermediate (RI), a partially double-stranded structure (Fenwick *et al.*, 1964). As far as is known, the cell does not possess a polymerase capable of using an RNA template for the production of RNA. Therefore, in order to form a key virus structure, the RI, a virus polymerase must be produced.

When the virus polymerase is present, the input virus RNA can be replicated. It is first employed as a template to form a complementary RNA strand, the so-called negative strand (Pollet *et al.*, 1967). The combination of the viral RNA with its negative strand is the replicative form (RF), a completely base-paired structure (Franke and Hofschneider, 1966). The negative strand of the RF may not employ a virus polymerase to produce its complement, the progeny virus RNA or positive strand. Therefore, once the RF has been formed, replication of virus RNA may

proceed on the RF by alteration of its base-paired structure to allow for the displacement of partially or completely formed positive strand RNA molecules by nascent RNA chains. Thus, tails of nascent RNA chains begin to appear. This new structure, a base paired molecule with tails of nascent virus RNA, is the RI. In some manner the RI is also employed to carry out virus protein synthesis, since virus polysomes contain the RI structure (Friedman, 1968b). This indicates that protein synthesis is initiated and perhaps carried out on nascent RNA chains.

The genome of Semliki Forest virus, an RNA of about 2×10^6 daltons (Wecker, 1959), probably contains genetic information for the synthesis of five proteins. Three of these are structural and two are non-structural. Early in infection all five of these proteins are produced, but late in infection only the three structural viral proteins are made (Friedman, 1968c). It is likely that the two non-structural proteins produced early are functional proteins, possibly enzymes. The mechanism for control of genetic expression in Semliki Forest virus infection is unknown but may involve one of the structural proteins acting as a repressor, as is the case with RNA bacteriophages (Nathans *et al.*, 1966).

The assembly of the infectious virus is intimately connected with its egress from the cell. First the virus RNA is assembled into the virus core (Friedman and Berezesky, 1967), a nucleocapsid containing RNA and two of the three structural virus proteins (Friedman, 1968c). This core structure then buds from the plasma membrane, picking up part of this as the external virus membrane (Acheson and Tamm, 1967). The outer membrane contains lipid, phospholipid, and an additional virus-specific structural protein (Strauss *et al.*, 1968). The complete growth cycle of Semliki Forest virus in primary chick cells takes about seven hours (Taylor, 1964). Figure 3 summarizes the growth cycle of Semliki Forest virus.

Vaccinia virus has a complex structure also containing an outer envelope and protein coat surrounding an inner core which consists of the DNA of the virus genome with its own protein coat (Westwood *et al.*, 1964). This structure contains about 20 distinct polypeptides (Holowczak and Joklik, 1967a). The DNA of vaccinia virus contains sufficient genetic information to code for about 400 proteins.

Vaccinia virus adsorbs to cells and is taken into the cytoplasm in phagocytic vesicles and the outer coat and membrane are lost, probably through digestion by lysosomal enzymes (Dales, 1963). The core particle containing DNA with a protein coat remains. Its uncoating is indirectly due to a virus RNA polymerase which is present in the structure of the virion (Kates and McAuslan, 1967b). This enzyme produces a virus

messenger RNA which in turn directs the synthesis of a virus protein, which functions to remove the protein coat of the core (Munyon *et al.*, 1967). The DNA genome of the virus is now freed to operate in the cytoplasm of the cell (Fig. 4).

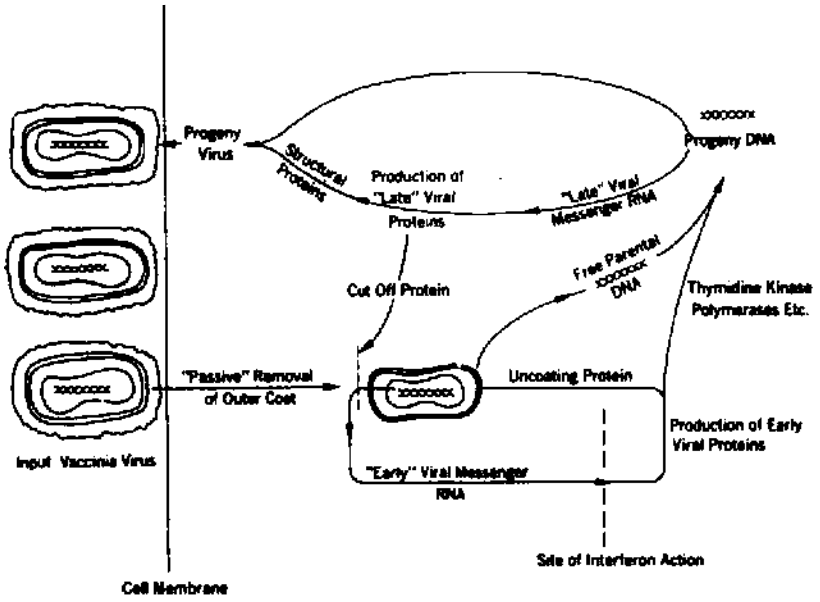

FIG. 4. Replication of vaccinia virus. Here the site of interferon action appears to be after the production of messenger RNA transcribed from the DNA of the input virus. Virus polyribosomes fail to form.

DNA synthesis then proceeds very rapidly, going to completion by about five hours after infection in HeLa cells (Salzman, 1960); the growth cycle of the virus, however, requires 16 hr (Joklik and Becker, 1965). The reason for this delay is indicated in studies of vaccinia virus messenger RNA and protein synthesis. Early in infection virus messenger RNA of about 12S in size is produced (Becker and Joklik, 1964). This RNA is probably produced by the RNA polymerase present in the virus structure; it contains the genetic information not only for the uncoating enzyme but also for virus structural proteins and enzymes responsible for virus DNA replication (Kates and McAuslan, 1967a; Holowczak and Joklik, 1967b). After DNA replication is completed the predominant virus messenger RNA becomes larger, about 20S in size (Becker and Joklik, 1964).

The new messenger RNA is produced on progeny virus DNA and is translated to produce both virus structural proteins and regulatory proteins (Holowczak and Joklik, 1967b; McAuslan, 1963). The regulatory proteins appear to suppress the function of the early (12S) virus messenger

RNA (Kit and Dubbs, 1965). The virus DNA, lipids, and structural proteins are then assembled into the progeny virus particles. The growth cycle of vaccinia virus is summarized in Fig. 4.

MECHANISM OF ACTION OF INTERFERON

Isaacs and his associates showed very early that interferon has no direct effect on viruses, for unlike antibodies, interferons do not neutralize viruses (Lindenmann *et al.*, 1957). However, cells treated for various periods of time at 37° with sufficient concentrations of interferon supported virus growth poorly or not at all. In cells incubated with interferon at 4°, antiviral activity failed to develop. Therefore, it was certain that an interaction between interferon and the cell must take place in order to render the cell resistant to subsequent virus infection. The nature of this cell–interferon interaction could best be studied by observing the activity of the virus in the interferon-treated cell. It is first necessary, however, to review briefly the interaction between interferon and the cell. Interferon, like many other biologically active polypeptides, binds to cells with which it is interacting (Friedman, 1967). This binding requires little or no energy as it takes place at 1°C. An equilibrium is established between cell-bound and free interferon in which almost all of the interferon (more than 95%) remains free and an almost undetectable amount becomes tightly bound to the cell (Buckler *et al.*, 1966; Levine, 1966). This equilibrium is established very rapidly even in the cold. The cell-bound interferon remains on the cell surface, at least at first, since cell-bound interferon can be destroyed by trypsin, resulting in an inhibition of the development of antiviral action (Friedman, 1967).

After binding the next series of steps which lead directly to an antiviral state in the cell are almost completely obscure. Several observations to be discussed below, however, may be fit together to form a somewhat coherent picture. Firstly, interferon does not appear to be consumed to any great extent in establishing an antiviral state, a not too suprising observation when it is considered that very little interferon is bound to the cell. Also, a period of pretreatment at 37° is necessary for the establishment of interferon action, suggesting that some metabolic activity is required (Friedman, 1967). Finally, the action of interferon is potentiated by cyclic-3′5′-adenosine monophosphate (CAMP) although this compound does not by itself mimic the action of interferon (Friedman and Pastan, 1969). Since CAMP has been shown to enhance the production of induced enzymes in bacteria (Pastan and Perlman, 1968), this finding may indicate

that interferon action is somehow related to the production of an induced protein.

Some insight into the nature of the activity which establishes the antiviral state in interferon-treated cells has been obtained in studies on the modification of interferon action by metabolic inhibitors. Inhibitors of cell RNA synthesis, especially actinomycin D, have been shown to block completely the establishment of an antiviral state in interferon-treated cells (Taylor, 1964). A requirement for cell RNA synthesis for interferon action is therefore firmly established. Other studies have employed amino-acid analogues (*p*-fluorophenylalanine) (Friedman and Sonnabend, 1964) or inhibitors of protein synthesis (Levine, 1964; Friedman and Sonnabend, 1965) in order to establish whether protein synthesis is also required for the development of interferon action. The published results have all indicated that protein synthesis is required for interferon action but the results are not as convincing as those establishing the requirement for RNA synthesis.

In summary, therefore, it appears that very small amounts of interferon are rapidly and firmly bound to cells. A series of events is then set off; these seem to require synthesis of cell RNA and protein in order for an antiviral state to be established in the cell.

Studies on virus function in interferon-treated cells have been carried out in whole cells and in cell extracts. Studies in whole cells clearly established that interferon does not affect the very early events in virus infection, such as virus adsorption, entry, and uncoating, since interferon treatment inhibited the replication of infectious viral RNA (Grossberg and Holland, 1962).

In studies of interferon action on Semliki Forest virus, the production of new virus RNA was inhibited by interferon treatment (Taylor, 1965). The production of the RF of the virus was less susceptible to the effect of interferon treatment than was the production of the RNA of the mature virus itself (Mecs *et al.*, 1967). This may have been due to the relatively small amounts of interferon used in this particular study. The greater inhibition of the later stages of virus growth may be related to a requirement for several virus proteins to reach these stages. The production of the RF may require only small amounts of one enzymatic protein, but the formation of the complete virion obviously requires the production of many structural subunits for each virion.

There is also an inhibition of the production of the virus RNA polymerase several hours after infection in interferon-treated cells (Sonnabend *et al.*, 1967). This finding may have indicated that there was an effect

directly on virus protein synthesis or (more likely) that accumulation of the virus enzyme was inhibited as a consequence of an inhibition of virus RNA synthesis. The results discussed so far suggest that the site of action of interferon must be on some early event occurring after uncoating of the virus, but before virus RNA synthesis is initiated.

Interest therefore centered about these early events and the fate of the parental strands of virus RNA. In the case of Semliki Forest virus some of the parental strands were noted to pass into a ribonuclease-resistant form, the RF or the RI or both forms (Friedman *et al.*, 1967). In cells treated with high concentration of interferon, as in the case of cells treated with inhibitors of protein synthesis, little or no ribonuclease-resistant RNA was generated. These results suggested that no virus polymerase was formed; this would account for a failure of the double-stranded RNA forms to appear—the negative strand could not be generated in the absence of the enzyme (Fig. 3).

Some substance was given to this notion on the basis of studies of protein synthesis directed by the input (parental) RNA of Semliki Forest virus. In the first hour of productive virus infection, six proteins were produced in infected cells (Friedman, 1968b). Of these six proteins, three are virus structural proteins, two virus non-structural proteins, and one is not virus at all, but cell in origin. In interferon-treated cells only the cell protein was produced. Moreover, in these experiments advantage was taken of the observation that synthesis of RNA by the virus is temperature-sensitive and does not take place at 42° (Ruiz-Gomez and Isaacs, 1963). In infected cells incubated at 42° virus protein synthesis, however, takes place at normal levels during the first hour of productive infection. This indicated that early in infection under conditions which inhibited virus RNA synthesis, virus protein synthesis was normal and, therefore, suggested that the virus protein synthesis taking place very early in infection was carried out for the most part under the control of the input virus RNA. In interferon-treated cells incubated at 42° only the single protein of cell origin was produced. The latter indicates that interferon treatment probably inhibits the translation of the RNA of the input virus (Friedman, 1968b).

Studies with another RNA virus, Mengo, a picornavirus, agree to some extent with those on Semliki Forest virus. Replication of picornaviruses is similar in some respects to that of Semliki Forest virus. Mengo virus infection causes a rapid and sharp cut-off of cell protein and RNA synthesis (Baltimore *et al.*, 1963). In interferon-treated cells the cut-off of cell RNA synthesis caused by virus infection is partially inhibited; however, inhibition of cell protein synthesis still takes place (Levy, 1964). Also, the

production of the Mengo virus RNA polymerase is inhibited in interferon-treated cells, as is the case with the analogous Semliki Forest virus enzyme (Miner *et al.*, 1966; Sonnabend *et al.*, 1967).

Detailed studies of the fate of input Mengo virus RNA suggested that this rapidly became associated with a ribosomal 40S subunit. This combination seemed to be a preliminary step in the formation of a virus polysome necessary to produce virus proteins (Levy and Carter, 1968). Interferon pretreatment of cells inhibited the formation of this virus RNA –40S ribosomal subunit combination. If this interpretation is correct, the findings correlate well with some of the studies in cell-free systems to be discussed below.

In studies of vaccinia virus infection normal entry and preliminary uncoating of the virus was found in interferon-treated cells (Joklik and Merigan, 1966). Vaccinia virus cores were produced but little or no uncoating of these took place (Magee *et al.*, 1968). As would be expected, since the virus polymerase is already present in the virion, early virus messenger RNA was produced; however, this RNA did not combine with ribosomes to form virus-specific polysomes. In both interferon-treated and untreated cells there was a marked inhibition of cell protein synthesis caused by disaggregation of host polysomes (Joklik and Merigan, 1966).

These findings can best be explained by two assumptions: (1) the inhibition of cell protein synthesis is due to a virus structural protein, since (2) transcription of the vaccinia genome takes place but translation does not. It should be noted that this latter assumption agrees quite well with the findings in studies of RNA viruses where translation of input RNA, which is equivalent to messenger RNA of a DNA virus, is also blocked (Friedman, 1968). The inhibition of cell protein synthesis by Mengo virus, on the other hand, probably does require some virus protein synthesis (Baltimore *et al.*, 1963) but only a few molecules of this virus product seem to be necessary to inhibit cell protein synthesis.

Studies on interferon action employing cell-free systems have attempted to find which element in the system necessary for virus RNA translation is altered by interferon treatment of cells. These studies have concluded that the ribosome is the site of interferon action; they suggest that interferon treatment somehow alters ribosome structure in such a way that virus RNA cannot attach to and/or be translated by such altered ribosomes. The exact mechanism whereby this might occur is obscure and there is disagreement about how the inhibition of translation is accomplished mechanistically. A suggestion has been that a translation-inhibiting protein (TIP) is produced in interferon-treated cells and then

attaches to ribosomes (Marcus and Salb, 1966). The same authors working with chick cells presented data that indicate that virus RNA was bound to ribosomes from interferon-treated cells with somewhat decreased efficiency but was not translated; other studies utilizing mouse cells imply that virus RNA simply does not attach to ribosomes from interferon-treated cells (Carter and Levy, 1969). It should be noted also that two other laboratories have been unable to confirm the above *in vitro* studies (Sonnabend and Lockhart, 1969). Also, results indicating that interferon may inhibit the growth of other intracellular parasites such as *Toxoplasma gondii* (Remington and Merigan, 1968), or *Plasmodium berghei* (Jahiel *et al.*, 1968), or TRIC agents (Hanna *et al.*, 1966), all of which have their own ribosomes, suggest that the host ribosome may not be the site of interferon action. It may in reality be somewhat premature at present to speculate on initiation of protein synthesis in animal cells as almost no information is yet available on this process.

A result with an adenovirus 7-SV 40 hybrid virus indicates that the structure of the nucleic acid of a virus may be important in determining whether antiviral activity is detected in an interferon-treated cell. Adenovirus 7 growth is resistant to interferon action, but SV 40 is fairly sensitive. Production of the SV 40 t-antigen by the hybrid, which appears to be formed by the integration of part of the SV 40 DNA genome into the adenovirus DNA genome (Baum *et al.*, 1966), is insensitive to the action of interferon (Oxman *et al.*, 1967). The meaning of these results depends on whether the messenger RNA transcribed by the adenovirus 7-SV 40 hybrid and responsible for the translation of SV 40 genetic information is a hybrid molecule. If this RNA is a hybrid of adenovirus and SV 40 messengers, the result may indicate that interferon treatment affects the ability of the cell to translate SV 40 messenger RNA by in some manner altering the ability of the cell to recognize the SV 40 RNA as an initiation site for protein synthesis. In the hybrid the initiator site might be on the adenovirus portion of the messenger.

If the RNA is not of a hybrid nature, the result may suggest that in the interferon-treated cell a specific portion of the input SV 40 virus genome is the point of attack of an interferon-induced protein. The attack might alter the nature of the product of transcription in a DNA virus or prevent translation of an RNA virus. In the case of the hybrid, the critical site on the SV 40 genome might be a part of the genome which is missing.

In summary, interferon is bound to cells and establishes an antiviral state by causing the production of RNA and possibly a protein which actually appears to mediate resistance. The locus of interferon action is the

translation of virus RNA, either the input RNA of an RNA virus or the messenger RNA transcribed by the input DNA of a DNA virus. The site of action of interferon in the cell has been postulated to be the ribosome, but other sites such as transfer RNA and messenger RNA binding factors remain as distinct possibilities. It is also possible that an interferon-mediated factor acts directly on virus messenger RNA. Whatever the actual locus of interferon action is, presently available data indicate that real differences must exist between the RNA of RNA viruses or the messenger RNA of DNA viruses, which are sensitive to interferon action, and the messenger RNA of the cell. The antiviral state established by interferon allows the cell to exploit this difference to the disadvantage of the virus. Figure 5 summarizes what is thought at present to be the mechanism of interferon action.

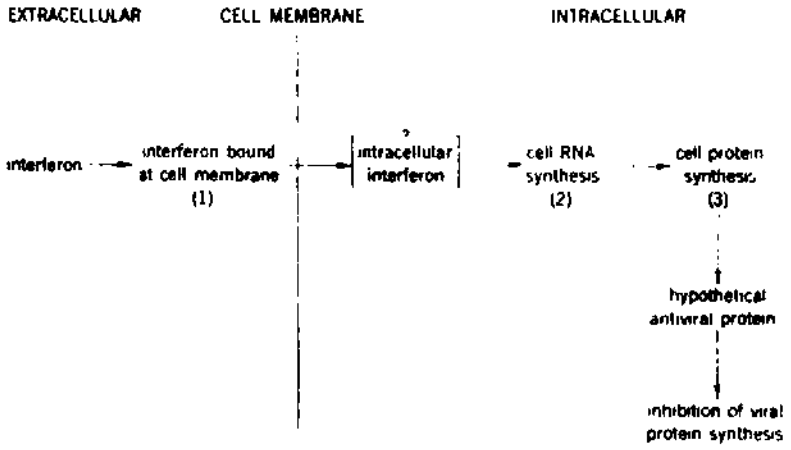

FIG. 5. Present concept of the mechanism of interferon action. Action may be blocked by (1) trypsin, after adsorption of interferon; (2) actinomycin D; or (3) puromycin or fluorophenylalanine before addition of interferon.

STIMULATION OF INTERFERON PRODUCTION BY VIRUSES

This section will be concerned for the most part with interferon production induced by viruses. Nonviral stimulation of interferon production and the heterogeneous nature of interferon will be discussed in another chapter.

The factors determining how much interferon will be stimulated by any virus-cell interaction are related to the virus and to the cell and its metabolic state. It was noted by Isaacs (1963) that in general viruses which were

good producers of interferon were also those which were sensitive to its antiviral activity. For instance, the following virus groups are listed in decreasing order of both sensitivity to interferon and ability to induce interferon in chick cells: arboviruses, picornaviruses, myxoviruses, poxviruses, and adenoviruses. At present it is not understood why this is so. Also some exceptions to this general rule exist; for instance, vesicular stomatitis virus is fairly sensitive to the antiviral action of interferon, but is a poor inducer (Baron *et al.*, 1967).

Viruses differ not only in their capacity to induce interferon production but also in the kinetics with which this production takes place. Semliki Forest virus and related arboviruses usually cause very rapid production of interferon (Friedman, 1966a), in some cases within a few hours of their inoculation into animals or introduction into tissue cultures. In the case of other viruses production is slower, sometimes coming after the end of virus growth (Wagner, 1964).

Different strains of Semliki Forest virus vary in their capacity to induce interferon (Finter, 1964a). This has been shown to be true of several other groups of viruses. Attenuated strains of viruses are in general better producers of interferon than are virulent strains of the same virus. Interferon may therefore be one factor in accounting for virus pathogenicity (Enders, 1960). In attempting to define exactly what factors are involved in interferon induction, it is of particular interest that virus strains apparently differing only in relatively minor ways may induce very different amounts of interferon in the same system. These findings may be related to the interaction between the virus and the cell (see below).

The infectivity of a virus preparation is a crucial factor in determining how good an inducer of interferon it will be. Some viruses are good inducers whether infectious or non-infectious (Gifford and Heller, 1963); others, only when they have been inactivated (Ho, 1964); and, finally, still others, only when infectious (Henderson and Taylor, 1961).

It is not clear whether interferon is induced by a structural element or by the replication process of a virus or by both. The discovery that double-stranded virus or laboratory produced RNA forms may induce interferon production leads to the interesting suggestion that this might be the common factor in the production of interferon; that is, the production of double-stranded virus RNA (the RI or RF) in the cell might be the stimulus for interferon production (Lampson *et al.*, 1967). In the case of Semliki Forest virus, for instance, it was shown that interferon was only produced under conditions which permitted the production of double-stranded virus RNA (Skehel and Burke, 1968).

It may not be true, however, that double-stranded virus RNA is the only inducer of interferon production, as it is certain that DNA viruses such as vaccinia virus are fairly active stimulators of interferon production (Isaacs, 1963). There is some reason to think that vaccinia virus contains or induces the production of a double-stranded RNA. However, single-stranded RNAs (Baron *et al.*, 1968) and such diverse substances as phytohemagglutinin (Wheelock, 1965) and other mitogens (Friedman and Cooper, 1967) and synthetic polyanionic copolymers (Merigan and Finkelstein, 1968) appear to be effective in inducing interferon production under some conditions. In addition it has not been completely ruled out that other structural elements or products of virus replication such as proteins or lipids might be inducers of interferon.

Many cell factors influence the yield of interferon in any system. For instance, when the interferon production of peripheral white cells is studied, all the interferon produced seems to come from lymphocytes; polymorphonuclear cells produce little or no interferon (Edelman and Wheelock, 1968). In tissue culture or whole animals, different cell lines (Lockhart, 1965) or different tissues (Kono and Ho, 1965) also may produce differing amounts of interferon. The S strain of HeLa cells is a very poor producer of interferon, while other HeLa strains have been reported to produce interferon (Cantell and Paucker, 1963a). Many other instances are known of strain, species, or tissue differences in the capacity of cells to produce interferon following a well-defined virus stimulus.

Interferon production in animals has also been studied in localized sites, in the whole animal, and in the circulation. Production in the respiratory tract following influenza infection (Isaacs and Hitchcock, 1960), in the brain following arbovirus infection (Hitchcock and Porterfield, 1961), and in the skin following vaccinia infection, among many other systems, have been well studied. Interferon production in these instances is usually more rapid than most other known humoral responses. Indeed, it is exceeded in rapidity only by the inflammatory response among the general animal reactions to infection. Studies on human interferon production agree in most respects with the generalizations made above about interferon production in experimental animals and cell cultures.

The metabolic state of cells has been found to influence very greatly the yield of interferon obtained in a given system. Most studies concerning these observations have been carried out in tissue cultures where the environment can be easily manipulated. For instance, the age of the cells in primary cultures very greatly affects the interferon response since older cultures appear to be much better producers than newly seeded cultures

(Ho and Enders, 1959). This may be a reflection of the general metabolic state of the cells, as older cultures have a decreased rate of macromolecule synthesis (Carver and Marcus, 1967). Interferon production has been found under some experimental conditions to vary inversely with the general rate at which macromolecular synthesis is being carried on in healthy cells (Friedman, 1966a).

Other factors have been implicated in the rate of interferon production, but these appear to be due basically to one of two causes. Firstly, many manipulations such as alterations in environmental temperature or of pH of the culture medium usually affect cell structure and function adversely (Hallum *et al.*, 1968). Interferon production might also be expected to be adversely affected. Secondly, manipulations which might inhibit the cytopathic effect of viruses, such as raising the incubation temperature of a temperature-sensitive virus strain, might be expected to increase interferon production, since the cell structure and function would be preserved for a prolonged period of time.

The latter point is of some interest as it stresses that the *interaction of virus and cell* in a system determines to a great extent the amount of interferon produced. A virus which has a great capacity for inhibiting cell macromolecule synthesis would not be expected to be a good inducer of interferon. This is why some viruses which rapidly inhibit cell RNA and protein synthesis (see below) are poor producers of interferon (Wagner and Huang, 1966).

The ability to inhibit cell protein synthesis may not, however, be a general property of a virus in the sense that this may not occur during all infections by this virus. Newcastle disease virus is usually non-cytopathic and an excellent stimulator of interferon (Henle *et al.*, 1959), but in chick cells most strains are markedly cytopathi cand also very poor interferon stimulators (Friedman, 1964). Other well-studied instances of somewhat similar systems have been recorded. These may be related to the phenomenon of inverse interference, a term used by Lindenmann (1960) to describe inhibition of interferon production by an inducing virus due to infection with another virus. The clearest instance of this phenomenon is the marked inhibition of the interferon production ordinarily induced with Newcastle disease virus in calf kidney tissue cultures by previous infection with parainfluenza virus 3 (Hermodsson, 1963).

The mechanism of action of several other inhibitors of interferon production is as yet unexplained. Well-controlled studies have indicated that some steroids (Kilbourne *et al.*, 1961) and hydrocarbon carcinogens (DeMaeyer-Guignard and DeMaeyer, 1965) depress interferon synthesis.

The mechanism of action of these substances is for the most part unknown, and it is not at all certain that interferon inhibition is related to the better known biological activities of these substances.

Some understanding of the biochemical events necessary for interferon production has been gained through studies with metabolic analogues and antibiotics. Studies employing fluorodeoxyuridine (Levy *et al.*, 1965b), iododeoxyuridine or aminopterin (Burke and Morrison, 1966) in doses which inhibited cell DNA, but not RNA or protein synthesis, showed that DNA synthesis was not required for interferon production. On the other hand, studies with actinomycin D, which blocks DNA-dependent RNA synthesis, have clearly shown that cell RNA synthesis is usually required for virus induction of interferon (Heller, 1963). The latter was a very important observation, since the dose of actinomycin D employed does not inhibit RNA synthesis by viruses like Semliki Forest virus, which do not require DNA dependent-RNA synthesis for growth. It is therefore certain that cell RNA synthesis is required for interferon production and that the genetic information for the production of interferon must be encoded in the genome of the cell. Studies with actinomycin D have suggested that the form of cell RNA necessary for interferon production is messenger RNA, but curiously no direct proof of this has been reported. Assuming that messenger RNA is the species required, in systems employing arboviruses the synthesis of messenger RNA for interferon is completed by about 4 hr after initiation of infection (Levy *et al.*, 1965a). The messenger RNA produced appears to be stable for at least several hours (Finter, 1966b).

Studies with inhibitors of protein synthesis such as puromycin (Wagner and Huang, 1965) or antimetabolites such as fluorophenylalanine (Buchan and Burke, 1964) have shown that interferon synthesis requires protein synthesis. This is not at all surprising since interferon is a protein, but the findings also suggest that production of proteins other than interferon itself, possibly enzymes, are required for interferon synthesis (Buchan and Burke, 1966).

Two other observations related to interferon production will be discussed. Because of some superficial similarities to phenomena in immunology, these have been termed tolerance and priming. Tolerance refers here to a refractory state soon established when repeated attempts are made to stimulate interferon. It has been observed both in tissue culture (Cantell and Paucker, 1963b) and in animal studies (Ho and Kono, 1968) and may be related to studies on priming. Priming refers here to a potentiation of interferon production by treatment with interferon (Isaacs and Burke, 1958) or inactivated virus (Burke and Isaacs, 1958) before actual

stimulation of interferon production. As in the anamnestic immune response, interferon is also produced more rapidly in the primed than in the unprimed system. Like interferon action, the primed state appears to require protein synthesis for its establishment (Friedman, 1966b).

The possible relationship between priming and tolerance may be that priming is a dose-dependent phenomenon. Very small doses of interferon (1 to 5 units) potentiate interferon production, but larger doses actually inhibit it, suggesting that the concentration of interferon in a system may in some manner regulate production, possibly by a feedback type of inhibition (Gerhart and Pardee, 1966).

In summary, interferon production stimulated by viruses is markedly affected by the nature of the virus and the cell system employed and by the metabolic state of these cells. It is uncertain at present whether a structural element of the virus or a product of virus replication is responsible for interferon induction. The virus–cell interaction is also of importance, since some viruses inhibit the metabolic activities of the cell. Of these activities, cell RNA and protein synthesis are required for interferon production. The interferon concentration present in a system may in some manner regulate the interferon production of the system. The mechanism of interferon production is shown in Fig. 6.

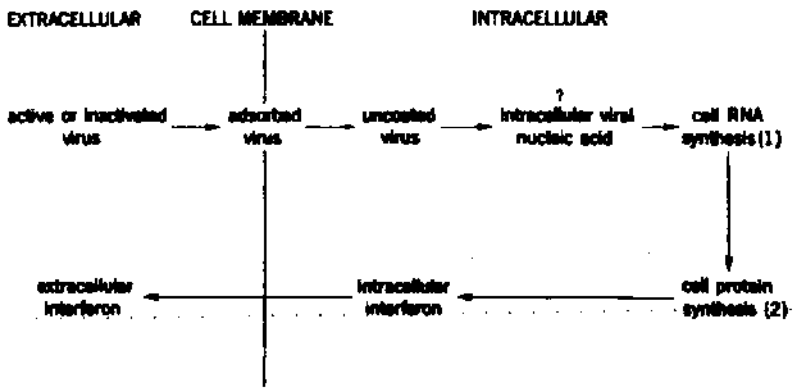

FIG. 6. Interferon production induced by viruses. Interferon production may be blocked by (1) inhibitors of cell RNA synthesis such as actinomycin D, or by (2) inhibitors of cell protein synthesis such as puromycin or metabolic analogues such as fluorophenylalanine.

INTERFERON AS A NATURAL INHIBITOR OF VIRUS INFECTIONS

The discovery of interferon, a natural protein with virus inhibitory properties, which is produced in response to virus infections, quite

reasonably led to the suspicion that interferon might be an important factor in natural recovery from virus diseases. One difficulty with such a notion was that antibody which was considered to be responsible for recovery from virus infections seemed to have a great deal to recommend it as such. Antibody is produced in response to virus infections or to vaccine administration; the presence of circulating antibody is well correlated with immunity to virus infections; passive transfer of antibody confers temporary immunity; and, finally, antiviral immunity was thought to possess the marked specificity of the antibody response.

At the time of the discovery of interferon three observations concerning antiviral immunity were unexplained. Antibody production was relatively slow as compared to both the rapid replication of viruses and the rapid onset and recovery seen in most virus diseases. Also, repeated observations suggested that recovery from virus infections was associated with a general, unspecific and short-term resistance to virus infections. Finally, the clinical disease agammaglobulinemia was discovered (Bruton, 1952). Patients with this condition had little or no circulating gamma-globulin and were extremely susceptible to many life-threatening bacterial infections; paradoxically, however, these patients usually recovered from most virus infections in a quite normal manner. Some had been vaccinated before the diagnosis of their condition.

The discovery of interferon seemed to provide a rational explanation for these observations. While there was no doubt that antibody was responsible for prevention of virus infection or reinfection, it seemed that interferon might be at least partly responsible for recovery from established virus infections, for recovery from virus diseases has two of the hallmarks of interferon activity: it is, like interferon production, extremely rapid and like interferon action, unspecific as far as its spectrum of antiviral activity.

Investigations of some viral diseases seemed to confirm that interferon played a major role in recovery from virus infections. In the case of vaccinia virus infection guinea-pigs with all of their measurable immune responses blocked by radiation and antimetabolites eliminated infectious virus and recovered in a normal manner. Interferon was produced at the local site of virus growth and the appearance of interferon was well correlated with a fall in titer of virus in the skin (Friedman *et al.*, 1962).

In the case of viruses which produce a viremia during the course of infection, production of circulating interferon, interferonemia, was found to be well correlated with the viremic phase of infection (Baron and Buckler, 1963). In many diseases associated with prolonged and severe

viremia, such as infection with Semliki Forest virus, encephalitis is the usual cause of death (Mims, 1964). Interferon production would on several counts appear to be an excellent mechanism for prevention of the development of encephalitis in just such a situation: interferon is quickly produced, and rapidly enters the circulation; also, interferon, because of its small size, can migrate into various tissues more quickly than can viruses (Fig. 7).

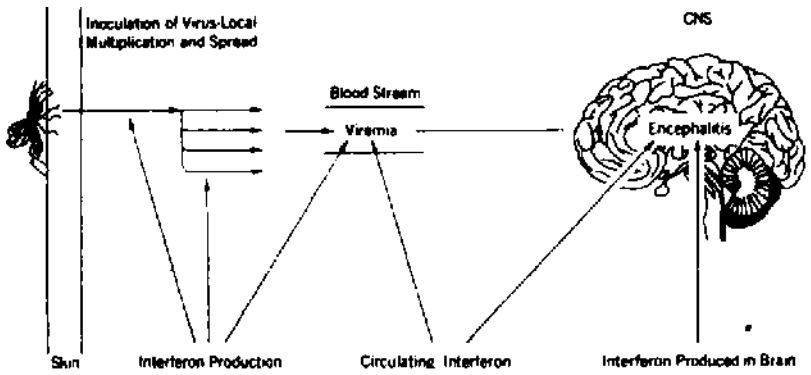

FIG. 7. Pathogenesis of an arbovirus infection and effects of interferon. Virus replicates at the site of inoculation finally resulting in viremia. The latter leads to an encephalitis. Interferon production is stimulated by local infection at the site of inoculation and in the brain and by the viremia.

Such findings as these made it seem reasonable to assign an important role to interferon in the process of natural recovery from virus infections. All the studies proporting to demonstrate this role for interferon have, however, lacked an important positive control—some indication that in the absence of interferon production the animal would not recover anyway. This is because no practical method has yet been found for inhibiting interferon production in a whole animal. On the other hand, in some studies in which antibody production was inhibited with antimetabolites and interferon production left unimpaired, an increased susceptibility of animals to virus infections was noted (Murphy and Glasgow, 1968). These results strongly suggest that in some virus infections antibody is an important factor in recovery.

The seemingly contradictory results of studies on the roles of antibody and interferon in recovery from virus infection may be resolved by suggesting that probably both have some part. In some infections antibody may be more important; in others, interferon may assume a major role. Other unspecific factors such as cell immunity, the febrile response and inflammation may also play major roles in different infections. Possibly, the particular virus involved, the locus of infection, and the physiological state of

the host in combination may influence which mechanisms are decisive in a given infection. In the case of interferon, however, its role as one mechanism of recovery from virus infections would appear to be well established.

Up to this point in this section interferon has been considered only as a mechanism of recovery from virus infections. It is also possible that in some cases interferon may prolong virus diseases and contribute to the establishment of chronic or persistent infections. Some excellent experimental models for this exist, one of the best studied being the establishment of a carrier infection by vaccinia virus in mouse embryo cultures. The rise and fall of virus titers in these cultures was inversely correlated with the rise and fall in interferon production by the culture. Destruction by trypsin of the interferon in the medium resulted in death of the cells. What appeared to be occurring ordinarily was a state of balance between virus and interferon in which the virus was unable to destroy the culture completely because of the production of interferon; however, the interferon produced did not protect the culture completely and virus was never eliminated. Destruction of interferon by trypsin shifted the balance in favor of the virus and resulted in death of the cultures (Glasgow and Habel, 1962).

Although no completely analogous situation has as yet been found in human or animal infections it is not unlikely that interferon could contribute to the establishment of a persistent infection. Persistent production of interferon by Burkitt lymphoma cells in culture, for instance, may indicate the existence of persistent infection by an as yet unidentified virus (Henle and Henle, 1965). Some chronic and progressively debilitating diseases may well be due to virus infections which are only partially controlled by cell factors such as interferon.

Infection of animals with tumor viruses is in a sense a chronic virus disease and several reports have appeared indicating a relationship between interferon and tumor viruses. Both RNA (Bader, 1962; Strandström *et al.*, 1962) and DNA (Allison, 1961) tumor viruses have been reported to induce interferon production and the growth of and tumor induction by these viruses, and to be sensitive to the antiviral action of interferon (Lampson *et al.*, 1963). In the case of polyoma virus an oncogenic variant was a poor inducer of interferon and a related non-oncogenic variant was a fairly good interferon inducer. It was uncertain whether interferon production was directly related to the prevention of tumors or whether it inhibited virus replication necessary for tumor induction (Friedman and Rabson, 1964). Other groups of polyoma variants have been isolated in which no such simple relationship was found between interferon production and oncogenic potential (Gotlieb-Stematsky *et al.*, 1966).

In a mouse tissue culture system interferon was found to inhibit morphological transformation and neoantigen production by SV-40 (Todaro and Baron, 1965); in cells already transformed by SV-40, however, prolonged treatment with interferon did not inhibit the production of the viral antigen (Oxman *et al.*, 1967). The meaning of the latter finding is dependent on whether the segment of SV-40 DNA which persists in these cultures is integrated into the cell genome and, as this seems likely (Sambrook *et al.*, 1968), on whether the SV-40 genetic information is translated as a polycistronic messenger with cell messenger RNA or as a small virus messenger RNA (see discussion above on the adenovirus 7–SV-40 hybrid).

In addition RNA tumor viruses and some leukemias caused by them have been shown to be sensitive to interferon both in tissue culture and *in vivo* (Gresser *et al.*, 1968). In the case of this group of tumor viruses the sensitivity is probably due to direct inhibition of virus growth by interferon, since the continued growth of malignant cells caused by the RNA tumor viruses may be related to the persistence of the inciting virus.

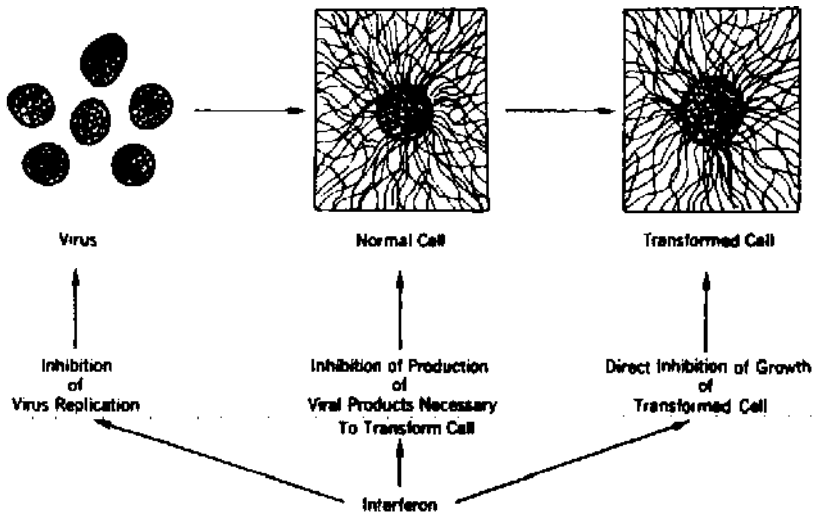

FIG. 8. Possible mechanisms of antitumor activity by interferon. In the case of virus-induced tumors interferon may inhibit virus replication or cell transformation directed by virus infection. Interferon might also directly inhibit tumor growth, although the latter is at present a conjectural notion.

Figure 8 illustrates three sites at which interferon might act on tumors induced by viruses. They are: (1) an inhibitory effect on virus replication; (2) inhibition of viral products necessary for cell transformation; (3) direct inhibition of the growth of tumor cells. The last is at present a theoretical

possibility, since no direct proof of such an action of interferon has been shown.

THERAPEUTIC AND PROPHYLACTIC USE

Although some studies have appeared indicating that interferon was ineffective in therapy or prophylaxis of animal or human virus infections (Scientific Committee on Interferon, 1965), a number of encouraging reports have also been published (Scientific Committee on Interferon, 1962). These have indicated that interferon may be useful in preventing or treating a wide variety of systemic virus diseases or localized virus infections—e.g. ocular vaccinia (Jones *et al.*, 1962), herpes (Park and Baron, 1968) or skin infections with vaccinia virus (Scientific Committee on Interferon, 1962). These reports have led to the hope that principles learned in studies with interferon can in some manner be applied to the treatment or prevention of human virus infections in general.

The problem of using interferon effectively in human infections will, however, be difficult to solve for several reasons. One of the imposing barriers to its use is the species specificity problem. The production by natural means of sufficient quantities of human interferon to be of general use now appears to be technically insurmountable, but possible indirect solutions do exist. For instance, studies on the structure and action of interferon may some day make possible the synthesis of an active interferon or interferon-like molecules in sufficient quantity to be of general use. Another approach might be to alter the chemical structure of animal interferons in such a manner as to make them active in humans. If this were possible, intact animal sources could be used. One final possibility along these lines might be the investigation of a wide variety of animal interferons in the hope of finding one with a chance cross reaction with human tissues. A precedent for this has previously been noted (Desmyter *et al.*, 1968).

A completely different tactic has also been attempted. This is the stimulation of autogenous interferon production by viruses or inducers of interferon, and in this manner very high concentrations of interferon may be reached in the tissues, indeed, much higher than could reasonably be expected by administration of exogenous interferon (Wheelock and Dingle, 1964). In this case the best approach at present would be to administer RNA or some other substance found to induce interferon production.

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In any event additional problems will be encountered in developing a useful form of therapy employing interferon. The time of administration of interferon relative to the time of onset of disease is a vexing problem but it seems certain that interferon will be potentially useful as a prophylactic agent. It has been used as such to inhibit Semliki Forest virus infections in mice (Finter, 1964b) and obvious applications of interferon administration in curbing epidemics of virus infections suggest themselves. The usefulness of interferon in established virus infections in general may, however, be limited. Once definite symptoms begin to appear in life-threatening virus infections such as encephalitides it may be too late to alter the course of the disease by administration of any quantity of interferon or of an interferon inducer. Even in tissue culture studies it certainly appears that cells which are virus infected cannot be saved by interferon treatment (Levy, 1964; Joklik and Merigan, 1966).

Some important exceptions to this general rule, however, suggest themselves at once. For instance, virus diseases in which the time of exposure is fairly easily established and a long incubation period exists may lend themselves to a combination of interferon prophylaxis and treatment. Important examples of this situation are rabies and hepatitis. It is possible that interferon-treatment might save a patient the discomfort and dangers of the usual course of therapy now employed in the prevention of rabies. In the case of relatively minor virus infections, especially those with prolonged courses, interferon may be useful in shortening the period of convalescence. This may apply to virus respiratory infections such as colds or pneumonias and such conditions as zoster where a long and painful course is the rule.

One objection to forms of therapy employing interferon should be listed, although at present it remains an entirely theoretical possibility. The effects of the recurrent administration of large amounts of inducers of interferon such as foreign RNA or indeed of homologous interferon itself are not established and as yet cannot even be guessed at. It is likely that these interferon inducers are entirely innocuous but other possibilities, however remote, do exist and it is reasonable to insert a note of caution in the consideration of possible studies of forms of therapy which may involve the genetic apparatus of the cell.

BIOLOGICAL SIGNIFICANCE OF INTERFERONS

However complex the mechanisms of interferon production and action may be, the presence of interferon in many animal species would suggest

that it is a useful and efficient system for contending with virus infections. All of the studies referred to so far in this review have been carried out in mammals or birds or in tissue cultures derived from them. A very wide variety of mammalian and avian species have been shown to produce and to be sensitive to the action of their respective interferons (Ho, 1962). Interferons or interferon-like substances have also been found in reptiles (Falcoff and Fauconnier, 1965) and fish (Beasley *et al.*, 1966). Moreover, antiviral factors which behave in some respects like animal interferons are found in extracts of virus-infected plants (Sela and Applbaum, 1962). One report has suggested that bacteria (*P. aeruginosa*) exposed to an inactivated bacteriophage produce an interferon-like substance (Mercer and Mills, 1960). Taken at face value, these reports suggest that production of, and response to, interferons or similar factors are indeed widespread biological phenomena.

Many aspects of what is known of the mechanisms of production and action of interferon are similar to well-established findings in other biological systems. For instance, interferons resemble many of the polypeptide hormones in chemical structure and molecular weight (Pastan, 1966). Like insulin, ACTH and TSH, interferon must be bound to have an effect on cells in order to induce a response (Pastan *et al.*, 1966); in this respect both interferons and hormones also resemble bacterial colicins (Nomura, 1964). Very little interferon or polypeptide hormone is consumed in carrying out their respective actions on cells (Buckler *et al.*, 1966) and again as in the case of many of the polypeptide hormones, interferon action appears to be involved in some manner with cyclic-3′5′-adenosine monophosphate (Friedman and Pastan, 1969).

Many aspects of the production and action of interferon are also reminiscent of induced enzyme systems in bacteria (Jacob and Monod, 1961). Indeed, what is known of the mechanisms of interferon production and action suggest that they may be part of a most unusual protein induction system, for while interferon itself seems to be an induced protein, its mechanism of action may involve the induction of yet another protein. Known systems which may easily be shown to be induced in animal cells are rare and potentially quite valuable to the investigator of animal cell biology. That the very same system should involve a remarkably stable extracellular protein which is an inducer itself would be most fortuitous. If a dual nature of interferon as an induced inducer really exists, interferon merits the attention of the biologist for far more than its antiviral activity.

The findings presented in the section on interferon production strongly suggest that in many cases interferon production truly involves the synthesis

of a new protein. While in some whole animal and tissue culture systems there is evidence that what has been called interferon induction is really a matter of release of preformed interferon (Finkelstein *et al.*, 1968), many other systems clearly involve synthesis of at least one new protein (Wagner and Huang, 1965). The most reasonable interpretation of these findings is that while enzymes which form interferon may also be induced, interferon itself is an induced protein.

In the systems in which preformed interferon appears to be released upon some stimulation, the nature of the preformed interferon is unknown. It may well exist as an interferonogen, an interferon precursor which is split to form a biologically active interferon. Many similar systems are well known in enzymology (Neurath, 1964), hemostasis (Ratnoff and Colopy, 1966), and the study of physiologically active amines (Kellermeyer and Graham, 1968), among other systems.

In the case of systems where interferon seems clearly to be an induced protein, the nature of the inducer remains at present unknown. The evidence at present favors virus or some non-virus RNAs as being one sort of inducer, but other virus substances such as proteins are by no means completely ruled out as inducers. Most of the non-virus inducers of interferon do have in common the property of being anions (Merigan and Finkelstein, 1968). The nature of the natural cell repressor, if it exists, is unknown. It is difficult even to speculate on the nature of a repressor which can interact with so many virus RNAs and with the other substances which induce interferon production, except to say that it is likely to be cationic.

One possible explanation for the many problems which arise about interferon induction involves a consideration of the products of this induction. Since the only assay for an interferon is a biological test of its antiviral activity, several substances with this activity may be produced by the cell. A given inducer may cause the production of one or more interferons; another inducer, the release of an active interferon previously stored as an inactive interferonogen. These different interferons have many similar chemical and physical properties and yet they differ in others, such as molecular weight, and may possess quite different chemical structures. The existence of a number of interferons to carry out what is in effect a single function may be analogous to the existence of a number of isoenzymes to carry out a single biochemical reaction (Cammarata and Cohen, 1950).

If the nature of interferon as an induced protein is not well understood, its possible role as an inducer is even more difficult to establish. In the section on the mechanism of action of interferon, evidence was discussed

which suggested that, for antiviral activity to appear after exposure of cells to interferon, cell RNA and protein synthesis are necessary. There is, however, some reservation concerning the latter. Moreover, even if both RNA and protein synthesis are necessary for interferon action, there is absolutely no information available concerning the nature of the RNA and protein being produced; that is, the protein might be either an enzyme or a structural component. Also, there are no data to suggest that there is necessarily an induced protein, and it is perfectly possible that the establishment of antiviral activity by interferon simply requires the continued synthesis of normal cell components, RNA or protein, which have a fairly rapid turnover rate. In this case the antiviral state established by interferon might be due to an interaction between small amounts of interferon and a cell factor (protein or RNA) which is normally present.

Finally, it is reasonable to question whether such a widespread biological phenomenon as interferon appears to be might not be of importance to the cell in some capacity other than its ability to generate antiviral activity. So far, studies on the effects of purified interferons on cells have failed to uncover any such function. As more is learned of complex biological processes, however, the interaction of various factors in such diverse systems as the inflammatory response, hemostasis, and complement activation have become manifest (Margolis, 1959; Webster and Ratnoff, 1961; Donaldson, 1968). It is possible, therefore, that interferon may yet be found to exercise some presently unsuspected control function in cells or to act as a co-factor in the general defense reactions of the organism.

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