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# The Continuing Search for Antiviral Drugs

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## I. Introduction

Although research into the mechanisms of virus infection is carried out by many sections of the scientific community, the search for antiviral drugs is almost exclusively the province of pharmaceutical manufacturers. The reasons for this are partly historical, but chiefly it is because the facilities for running large-scale screening programs are expensive and can only be met by commercial and, occasionally, governmental resources. Because of the need to protect their discoveries from unauthorized exploitation, a good deal of secrecy inevitably surrounds the work being carried out in commercial organizations. This secrecy is regrettable but necessary, since the survival of such organizations depends largely then on getting a fair financial return on the money invested by them in research. The security aspects of commercial research naturally restrict frank and constructive discussions between competitors as well as third parties, and this has been particularly true in antiviral research.

Perhaps it was a revolt against this enforced isolationism which led, at least in part, to the first Conference on Antiviral Substances held by the New York Academy of Sciences in 1965, in which manufacturers disclosed many of the details of their antiviral research and had a chance to discuss their failures and comparative successes (Whipple, 1965).

Since then there have been a number of reviews of progress in the field of antiviral chemotherapy, and in most of these there have appeared comments and recommendations which indicate that a radical rethinking is taking place of the prospects for antiviral drugs (Osdene, 1967; McFadzean, 1969; Goz and Prusoff, 1970; Swallow, 1971).

It was natural in the early 1950s to expect that antiviral drugs would be discovered which would be analogous to the antibacterial antibiotics, the darlings of the previous decade. The experience up to date has proved that this was not to be the case, and despite prodigious efforts by the drug houses, only three clinically useful antiviral agents, which are far from perfect, have emerged.

I believe there are lessons to be learned from this disappointing record, and I hope that the following remarks may help to continue further the critical reappraisal of this field, so that future progress may be faster.

## II. Technical Feasibility of Treating Virus Diseases with Drugs

Before embarking on a search for antiviral drugs, a number of points must be considered in order to assess the technical feasibility of treating or preventing a virus disease with a drug. Too often in the past massive screens have been set up against many viruses in the hope that a drug would turn up, and it would then find a natural place in human or veterinary medicine. Although it is true that random screening still offers the best chance of discovering new drugs, unless a realistic appraisal of the whole project is made at the outset, the products of random screens could well be useless as potential medicines.

Some of the more important considerations are listed below.

### A. Diseases of Economic Importance

In order to be commercially viable a drug must sell in sufficient quantities to pay for its development and manufacture as well as for future research. Because of this, and because antiviral chemicals tend to inhibit specific viruses, diseases of low incidence or low economic importance are ruled out as primary targets for drug development. This may seem inhumane, particularly in the field of human virus diseases, but the fact remains that it is no easier to find a drug against rabies than against influenza, and the sales from a specific antirabies drug would never cover its own development costs. Diseases of low incidence or low economic importance, no matter how serious the outcome may be for the infected individual, inevitably

must remain the subjects of sponsored research. Nevertheless, treatments for some of these diseases may emerge as drugs are developed against major diseases.

### B. Immunological Control

Many virus diseases, both of humans and animals, have been successfully controlled by vaccines. These successes have naturally led to improvements in the spectrum and duration of protection offered by vaccines until, today, it is difficult to see how antiviral drugs could compete with vaccines in the control of many virus diseases. As examples one may cite smallpox, yellow fever, polio, and, more recently, measles among human diseases, and Newcastle disease, Marek's disease, and infectious bronchitis among poultry diseases—an area of veterinary disease control where vaccines have been particularly important.

Despite these triumphs, vaccines are not, and probably never will be, the complete answer to the control of certain virus diseases. It is in these areas where drugs would be useful, and it is on these diseases that efforts should be concentrated. The two most important human diseases in this class are influenza and the common cold.

When a novel strain of influenza appears among the human population, as happened in 1933, 1947, and 1956, existing immunity to the previous current influenza strain is not effective, and widespread epidemics of disease occur. The disease spreads so rapidly after it first appears that it is not possible to develop, distribute, and administer a vaccine based on the new strain soon enough to protect useful numbers of the population. Even after the initial overwhelming pandemic, successive epidemics will occur as the disease penetrates into pockets of the community that had escaped infection. The disease is then maintained partly by the continuing appearance of susceptible juveniles, partly by spontaneous antigenic modifications in the virus enabling it to overcome previous immunity, and partly by the general decline in immunity of other individuals with the passage of time. After the initial pandemic, it is theoretically possible to control the disease with widespread vaccination, but in practice this is not done. Consequently the disease smoulders on in the community, appearing as isolated cases and occasional outbreaks and epidemics. These are the conditions under which the disease normally exists, and it is this situation, rather than the much publicized pandemics, which causes the greatest economic loss to industrialized countries. The overall loss in 1956–1957 in Great Britain due to the Asian influenza pandemic was estimated at £100 million, but the continuing loss, from that time on has been at least £30 million each year. Losses in Europe, North America, Japan, and similar industrialized communities must be

comparable, and if only a fraction of the disease could be prevented by a drug, the economic benefits would be enormous.

Losses due to the common cold are comparable. The careful study of Lidwell and Williams (1961) showed that approximately $11 \times 10^6$ working days are lost each year in Great Britain alone from the common cold. Nevertheless, the prospects for a common cold vaccine are poor because of the large number of viruses which are known to cause the disease. There are 89 known rhinoviruses (Kapikian, 1971), probably a comparable number of as yet unclassified rhinoviruses, a growing catalog of coronaviruses (Kapikian, 1969), and a selection of other viruses including myxoviruses, adenoviruses, and herpesviruses (Tyrrell, 1965), all of which have been isolated from clinical colds. It is the serological diversity of these etiological agents which makes the prospects for a vaccine so poor, and the common cold must, therefore, be considered as a target, even though a difficult target, for antiviral drugs.

Besides the problems of antigenic variation, exemplified by influenza, and the multiplicity of serotypes, exemplified by the common cold, there are two further problems associated with the control of respiratory diseases by parenterally administered vaccines. The first is that circulating antibodies appear in only small amounts in respiratory mucus, and, consequently, the degree of protection afforded to the respiratory tract is less and of shorter duration than might be expected. The second problem has been brought to light by the use of experimental vaccines against respiratory syncytial virus disease in infants. When infants who had been vaccinated parenterally against this disease contracted the natural disease, they were more ill than infants who had not received the vaccine. The reason seems to be that the circulating antibodies resulting from parenteral vaccinations not only offer little protection to the respiratory tract, but when a natural infection occurs, these antibodies combine with the virus antigens at the surface of the respiratory epithelial cells causing an inflammatory response with a corresponding increase in the severity of clinical symptoms (Chanock *et al.*, 1968; Kim *et al.*, 1969; Kapikian *et al.*, 1969). Chanock *et al.* (1970) have pointed out that if an immunopathological process involving serum antibodies occurs during respiratory syncytial virus infection, then stimulation of local, respiratory tract, secretory antibody by intranasal instillation of live or inactivated virus may give adequate protection without unwanted hyperreactivity.

A similar allergic reaction between virus antigen and preexisting antibody is a factor, possibly a major factor, in the pathological processes initiated by herpesviruses (Jones and Patterson, 1967) and marks the herpes diseases as possible candidates for antiviral drug development.

### C. Dosing the Host

One factor that deserves more careful consideration than it usually receives is the way in which a potential antiviral drug would be administered to the animal or patient requiring protection. Clearly a common cold treatment would be unacceptable if it had to be given intravenously 3 times a day. But there are less obvious, but no less real, difficulties in dosing large herds of cattle or sheep so that effective protection is maintained. With free-ranging animals the duration of protection from a single dose would need to be prolonged to offset the labor of administering the dose. Also, it is easier to dose herds by injection than by mouth, so that certain veterinary antiviral drugs may not be required in an orally active formulation. Conversely, oral dosing, preferably by an addition to food or drinking water, is the most convenient way of dosing poultry.

The onset of symptoms in most virus diseases is acute and may be the first indication that the host has contracted an infection. Because of this, it is usually assumed that antiviral drugs will only be of value in preventing and not in curing virus diseases. Nevertheless, although the periods of virus growth in infected individuals may be short, they may well be long enough to allow useful therapy. For example, Patel *et al.* (1964) have shown in volunteers infected intranasally with Coxsackie A21 virus that maximum virus growth precedes the onset of symptoms by 24 hours. But from their work it may be seen that a considerable amount of virus growth is concurrent with the period of overt symptoms, and application of antiviral drugs during this period of 2–3 days might well prevent the full development of the disease. The work of Douglas *et al.* (1966) with volunteers shows that a similar period exists in acute rhinovirus infections, and Dawkins *et al.* (1968) and Wingfield *et al.* (1969) have shown that the course of influenza in humans can be modified, even after the onset of symptoms, by treatment with 1-aminoadamantane.

### D. Other Aspects

It hardly seems necessary to point out that before undertaking a search for a drug against a particular disease, the etiological agent of the disease should be unequivocally identified.

There are a number of important virus diseases for which the causative agent is either unknown or is in doubt, for example, bovine pneumonia and human epidemic viral gasteroenteritis. It would be a mistake to set up screens against agents that were only suspected of being implicated in these diseases in case subsequent work should demonstrate that these were not in fact the causative agents.

Although it is difficult to predict changes in governmental legislation toward public or animal health, nevertheless, the existing and prospective legal position in various countries should be considered since these may affect the prospects for potential drugs. For example, in Great Britain, foot and mouth disease is controlled by the policy of slaughter and compensation, but in other countries the disease is controlled by vaccination. In the latter countries, a drug may find a ready market, but if legislation should change, then that market would be lost.

In summary, we may say that before embarking on a search for antiviral drugs, the target disease must be carefully selected by a consideration of all the relevant factors. The more important of these are:

1. There must be an adequate market for the drug.
2. There should be no effective immunological control, and no prospects for such control.
3. Due regard should be given to the practicability and the timing of dosing the patients or animals at risk.
4. The etiology of the disease should be clearly established.
5. Other relevant factors, e.g., medical or veterinary legislation, should be taken into consideration.

In Table I, a number of virus diseases of economic importance are listed together with some comments to illustrate how many seemingly attractive drug-target diseases are in fact precluded by other factors.

## III. Design of Antiviral Screens

It is easy to list the properties of the ideal antiviral drug—wide spectrum of activity, nontoxic, accessible to the target organ, etc. What is not so easy is to predict what sort of properties one might expect from antiviral leads detected by screening programs. All the same, it is only by intelligent attempts to do just this that screens may be designed to detect compounds that one day may lead to the development of useful medicines.

### A. Tissue Culture Screens

#### 1. *Design*

The scientific literature abounds with reports of antiviral chemicals discovered by screening random compounds in tissue culture systems, but all too frequently these compounds turn out to be false positives or active only against some relatively unimportant virus. For the products of a tissue

culture screen to be of potential value, the screen must meet the requirements outlined below.

First, the screen should be able to process large numbers of chemical compounds, or fermentation products, since the more that are tested, the greater the chance of success in finding active leads. Perhaps the time will come when new drugs can be designed and synthesized on entirely rational grounds, but at present most active leads are chance discoveries. Buthala (1965) quotes 6% of all compounds tested in a tissue screen as showing some antiviral activity, but in my experience the rate varies from 1 to 0.01%, the higher rate referring to influenza A viruses, and the lower rate to picornaviruses. Bauer (1967) has suggested that the larger the virus, the more susceptible it is to inhibition, since, for any given intracellular concentration of drug, a large virus will encompass, both physically and in terms of synthetic requirements, more drug molecules than a small one. Undoubtedly, this principle will contribute to our observed high rate of inhibition of influenza virus, but another important factor seems to be that the adsorption of influenza viruses to cellular receptors is particularly vulnerable to interference by extraneous substances.

Given that only a fraction of a percent of all compounds tested in tissue culture will show activity and that of these only a small proportion will show activity in animal models, a realistic screening rate would be not less than 5000 compounds a year. At rates less than this, the chances of finding useful compounds become so small as to make the whole project not worthwhile.

In many of the published screening procedures, the virus with which the screens are run seem to have been chosen more for the ease with which they can be handled rather than for their relevance to any virus disease target. For example, Ehrlich *et al.* (1965) describes a screen where the primary test viruses include parainfluenza type 3, measles, and poliovirus, and Johnson (1965) describes a screen that includes pseudorabies, adenovirus III, and mouse hepatitis virus. Of course, if wide-spectrum leads appear, the choice of test virus may be irrelevant, but the antiviral compounds (as distinct from interferon inducers) known at present are characterized by their relatively limited spectrum of activity, e.g., methisazone is active only against poxviruses (Bauer and Sadler, 1960) and possibly adenoviruses (Bauer and Apostolov, 1966); 1-aminoadamantane is active only against influenza $A_1$ and $A_2$ and not against other myxo- or paramyxoviruses (Davies *et al.*, 1964); guanidine and $\alpha$-hydroxybenzyl benzimidazole are active only against picornaviruses and not against other small ribonucleic acid (RNA) viruses (Eggers and Tamm, 1961). Thus, whenever possible, the viruses used in routine screens should be those that are responsible for the clinical

TABLE I

CHEMOTHERAPEUTIC VERSUS IMMUNOLOGICAL CONTROL OF VIRAL DISEASES

| Disease | World-wide importance | Etiological agent | Comments |
|---|---|---|---|
| Human | | | |
| Poliomyelitis | Declining | Identified | Successful control by vaccines and improved sanitation |
| Common cold | Great | Rhinoviruses<br>Coronaviruses<br>Adenoviruses<br>Myxoviruses | Multiple causative agents offer little prospect of effective vaccines; disease therefore must be considered as a drug target |
| Yellow fever | Declining | Identified | Successful control by vaccines and elimination of vector |
| Smallpox | Moderate | Identified | Disease eradicated in many areas by vaccination |
| Influenza | Great | Identified, but subject to change | Vaccines do not control the current serotype and will probably be useless against new serotypes A wide spectrum drug would be most valuable |
| Mumps<br>Measles<br>Rubella | Declining | Identified | Results with vaccines suggest that these diseases will eventually be well controlled |
| Herpes simplex and zoster | Moderate | Identified | Disease recurs in individuals with antibody Cutaneous, ocular, and encephalitic disease probably best controlled by drugs |
| Serum and infectious hepatitis | Moderate | In doubt | — |
| Epidemic gasteroenteritis | Moderate | In doubt | — |

| | | | |
|---|---|---|---|
| Cattle | | | |
| Foot and mouth disease | Great | Identified | Vaccines permitted in some countries; in others, disease controlled by slaughter Both methods have drawbacks, but it is difficult to see how drug protection could be maintained on large free-ranging herds |
| Rinderpest | High | Identified | |
| Virus diarrhea | Moderate | Unidentified | Until the etiology of these diseases is better understood, it is not possible to develop antiviral drugs, although such drugs would probably be valuable |
| Calf pneumonia | High | Unclear | |
| Pigs | | | |
| Swine fever | High | Identified | Vaccines used in some countries, slaughter in others A drug would be a practical proposition particularly as intensive farming methods increase |
| Transmissible gastroenteritis | Moderate | Identified | Vaccines at present poor, but likely to improve and be very effective |
| Poultry | | | |
| Newcastle disease | Great | All identified | Good control with vaccines. Live virus vaccines administered in drinking water or by aerosol now being introduced |
| Infectious bronchitis | High | | |
| Fowl pest | High | | |
| Marek's disease | High | | |
| Sheep | | | |
| Bluetongue | High | Identified | Vaccines permitted in some countries; in others, strict import regulations operate to prevent introduction of disease A drug would be difficult to administer to large flocks of free-ranging sheep |

disease, even if this means overcoming considerable technical difficulties associated with their growth and assay.

Large-scale screens use large numbers of tissue culture cells, and there has been an inevitable trend toward the use of continuous cell lines in screening procedures. Such cells have many attractive features. They grow rapidly, they can easily be obtained in large quantities, they remain "the same" year after year, they can be made to perform useful technical tricks such as rapidly changing the pH of their medium and surviving for long periods under agar, and, perhaps most important, they will support the growth of a wide range of viruses. Continuous cell lines seem to be the natural choice for running routine screens. Nevertheless, it is worthwhile remembering that many of the desirable technical properties exhibited by these cells may be a direct result of their neoplastic nature, and to use a continuous rather than a primary or diploid cell in a tissue culture system is to take yet another step away from the natural disease. It is true that antiviral agents, such as 1-aminoadamantane and methisazone, which can be shown to protect humans against virus diseases, also exert their antiviral action in neoplastic cells in tissue culture, for example HeLa and KB cells, but we have striking evidence that this may not always follow. We have recently discovered a family of chemical compounds that have high activity against rhinoviruses when grown in human diploid lung cells, but virtually no activity against the same viruses growing in monkey kidney cells, HeLa cells, or KB cells (Bucknall, unpublished results). These compounds and any others that may exhibit this property would have been missed in tests carried out in continuous cell lines.

In summary, a tissue culture screen should be able to process large numbers of test compounds, using viruses as relevant as possible to the diseases for which a drug is required, and should employ normal rather than neoplastic cells. Unfortunately, in most of the published screening procedures the last two requirements have been sacrificed to technical considerations designed to increase the number of compounds tested, as the following descriptions will show.

In its simplest form, a test for antiviral activity involves treating cultures of cells with a range of concentrations of a test compound. First the maximum concentration tolerated by the cells is assessed; then, second, the growth of virus at lower concentrations of compound that are not cytotoxic is measured. Several ingenious methods have been devised for measuring these two responses—cytotoxicity and virus growth—all designed to facilitate the screening of large numbers of compounds. For example, Herrmann *et al.* (1960) devised a zone-inhibition test in which large flat dishes of chicken cells were infected with test virus, overlaid with agar containing a

vital stain, and paper discs impregnated with test compounds placed on the surface of the agar. Compounds with antiviral activity showed two concentric zones around the paper disc, the innermost being pale in color due to the destruction of host cells by cytotoxic concentrations of compound diffusing from the disc. Outside this was a deeply staining zone where cells were exposed to nontoxic concentrations of compound which also protected them from the destructive effects of the virus with which they had been infected. Beyond this, where the concentration of compound was too low to protect the cells, the cell sheet was destroyed by virus and stained poorly. Thus, by visual inspection of the dishes after 3 to 4 days, active compounds could be quickly detected.

Rada *et al.* (1960) devised a similar agar diffusion test in which test compounds were applied to the virus-infected cell sheets in circular wells in the agar overlay.

Although agar diffusion tests are capable of processing large numbers of test compounds, they suffer from two drawbacks. First, they are of comparatively low sensitivity in detecting both the cytotoxic and antiviral levels of compounds, and second, they are limited to viruses that produce plaques under agar.

Rightsel *et al.* (1956) devised a system based on the fact that if cells were damaged either by the toxic effects of a chemical compound or by virus growth, they would not swing the pH of their medium. Thus, by incubating virus-infected cells in a series of concentrations of a compound and then looking for the cultures that had changed the color of the phenol red indicator in their medium from pink to yellow, active compounds could be detected. This technique has also been used to assay neutralizing antibody and interferon action (Pauker, 1965).

Finter (1970) described a system whereby the cytotoxic effects of test compounds could be assayed by the reduction in the amount of neutral red taken up by treated cells, and, similarly, the cytopathic effects of virus growth could be quantitated by measuring the reduction in the uptake of neutral red by infected cells. The system can readily be adapted to the screening of test compounds for antiviral activity. If myxoviruses are used in this system, because their cytopathic effects may not be pronounced, their growth is best monitored, not by a reduction in neutral red uptake, but by a quantitative hemadsorption method which matches the neutral red uptake method in its accuracy and sensitivity (Finter, 1964). In contrast to the zone-inhibition and pH-swing tests, the neutral red uptake test is precise in operation and may be used to demonstrate fine differences in the relative toxicity and activity of test compounds; it is probably no more time-consuming than the former tests.

A system of testing for antiviral agents based on the inhibition of nucleic acid synthesis was described by Miller *et al.* (1970) and has been used to screen compounds and mold metabolites for antiviral activity (Miller *et al.*, 1968). For the test, HeLa cells were suspended in a medium containing uridine-$^3$H. If a test compound has toxic effects on the HeLa cells, then the cellular RNA synthesis, as measured by uridine-$^3$H fixation, will be reduced. Similarly, if cells are infected with an RNA virus and treated with actinomycin D, then RNA synthesis will be due to virus growth only. Thus, if test compounds reduce this virus-directed RNA synthesis at concentrations that do not affect cellular RNA synthesis, then the compound is exerting a specific effect on virus growth. The test can also be used for deoxyribonucleic acid (DNA) viruses, the cellular and virus DNA synthesis being monitored by including thymidine-$^3$H in the medium. Virus DNA synthesis is distinguished from cellular DNA synthesis by disrupting the cells at the end of the test and treating them with deoxyribonuclease when encapsulated virus DNA is resistant to digestion and the unprotected host cell DNA is not. Thus, the selective effect of test compounds on the synthesis of virus DNA can be measured. The authors claim that the system operates satisfactorily with a range of viruses—some of them important disease organisms—and is simple, reliable, and rapid. The chief criticism of this method is that, because nucleic acid synthesis is used as the sole measure of virus growth, test compounds that might act on subsequent stages in the virus replicative cycle may not be detected. For example, any disturbances in the sequencing of virus nucleic acid, inhibition of structural protein synthesis, or failure of assembly or release of mature virions, would provide a sound basis for a useful drug, but these phenomena may not be detected in this type of test. Also, since high infecting doses of virus are used to give satisfactory operation of this test (up to 15 virus particles per cell), the test may be rather insensitive in detecting antiviral activity. All antiviral activity is, in the broadest sense, competitive, either at the level of cellular membrane receptors or at an enzymic or template level. Thus, the more virus is used to initiate infection, the less effective an antiviral compound is likely to be. Although this factor will not turn a highly active compound into an inactive one, it may well obscure low levels of activity which might be useful starting points for chemical exploitation. The real value of Miller's test is the use of the important biochemical system of nucleic acid synthesis to monitor the toxic manifestations of test compounds. This subject is discussed further in the following section.

### 2. *Assessment of Compound Toxicity in Vitro*

Reference has already been made to four methods for measuring the toxicity of chemical compounds in tissue culture cells: direct cytopathic ef-

fects, vital dye uptake, metabolic activity (pH-swing), and nucleic acid inhibition, and there is no shortage of other methods. Nevertheless, the inadequate assessment of compound toxicity in antiviral testing probably gives rise to more false leads than any other single cause.

Before discussing how compound toxicity might be measured, it must first be defined. Strictly, any interference with cellular metabolism by an extraneous compound is a toxic effect, and the most stringent tissue culture test of lack of toxicity is the continued normal division and growth of cells in the presence of an extraneous compound.

However, this test is too cumbersome for use in rapid screening procedures, and simpler, but less critical, tests are invariably used in primary screens.

Undoubtedly, the simplest method of assessing compound toxicity is by direct microscopic examination of cells for cytopathic effects or more subtle morphological changes. It is necessary for the observer to be trained to detect such changes, and an arbitrary scale must be devised to record the observations, but if these simple requirements are met, the method is generally successful. It may be objected that this system would be unworkable where large numbers of compounds are being screened because of the correspondingly large numbers of microscopic examinations required; but given a good low-power microscope, an experienced reader, and the fact that most random compounds tested will show no antiviral activity and will, therefore, not require more than a cursory examination, the system is reliable, fast, and economical. In the author's laboratory a system of this kind has been in use for over 5 years, and it is possible for one worker to screen a hundred compounds against three viruses each week. We have found that with this system, compounds appear to be toxic at lower concentrations than with either the zone diffusion or the dye uptake method. We conclude, therefore, that our method is more sensitive than the others mentioned in detecting the toxic effects of compounds.

The direct microscopic assessment of toxicity is not without its deficiencies, but if the method is seen only as a preliminary determination of toxicity, these deficiencies are not serious. Chief among these (and this applies even more to indirect methods) is the occasional failure to detect certain types of toxicity. For example, from time to time we have had compounds which appeared to prevent virus growth and to show no toxicity to confluent sheets of tissue culture cells. These cultures looked normal for several days in the presence of the compound, but viruses would not grow in these cells. Nevertheless, further studies (see below) have shown that the compounds were exerting an inhibitory effect on some aspect of the cellular metabolism and it was this which prevented virus growth.

We have investigated this effect with (*a*) inhibitors of nucleic acid synthe-

sis and (*b*) uncouplers of oxidative phosphorylation, two classes of compound that are particularly prone to giving misleading results. Nucleic acid inhibitors are often slow to produce cytopathic effects in confluent monolayers of cultured cells. The DNA synthesis of such cells is low, and sufficient RNA synthesis is often maintained in the presence of partially effective concentrations of an inhibitor, enabling the cellular structural integrity to be sustained. All the same, an invading virus is unable to replicate in a cell under these reduced circumstances, and this will lead to an apparent antiviral specificity. This is the mechanism by which the chlorinated ribofuranosylbenzimidazoles exert their antiviral effects (Bucknall, 1967). These compounds were extensively studied as antiviral agents before their "activity" was found not to be specific for the virus (Tamm *et al.*, 1954; Tamm and Nemes, 1957; Tamm and Overman, 1957).

Uncouplers of oxidative phosphorylation also often appear to be antiviral agents because concentrations that greatly reduce the energy-generating systems of cells in confluent monolayers are often slow to produce morphological changes. In this half-poisoned state, the cultures appear normal, but do not support virus growth, and thus another false "lead compound" is generated.

As mentioned earlier, these remarks apply to all tissue culture systems to a greater or lesser extent, and tissue culture tests for antiviral activity must always be regarded as strictly preliminary. Active leads from such tests must always be subjected to the closest scrutiny to determine whether the activity is truly specific for a virus-coded process or simply results from a subtle toxic effect on the host cell.

The margin between the maximum nontoxic concentration and the minimum antiviral concentration of a test compound is conveniently expressed as therapeutic ratio = max. nontoxic concentration/min. antiviral concentration and will vary according to how these two concentrations are determined. The simplest and most stringent test of the maximum nontoxic concentration of a compound *in vitro* is to grow cells in the presence of the compound and determine the maximum concentration at which division and growth will proceed normally. If this concentration, and lower ones, protect the cells from virus attack, then this is an unequivocal demonstration that the compound is exerting a specific effect on some aspect of virus replication.

Like Miller *et al.* (1970), we have found the inhibition of cellular nucleic acid synthesis, particularly RNA synthesis, to be a useful system for detecting the toxic effects of test compounds. Cells are treated with a range of concentrations of a compound, then the uptake of uridine-$^3$H into acid-insoluble material is measured and compared with that of normal cells

(Bucknall, 1967). The test is simple to run, and since the nucleic acid metabolism is a cardinal area in the cellular metabolism, even if a compound has no direct effect on the nucleic acid synthesis, disturbances of other synthetic or homeostatic mechanisms are quickly reflected in changes in the synthesis of RNA or DNA or both.

In Fig. 1, the dose–response curves of one experimental compound are determined in human diploid lung cells by the three methods outlined above—direct cytopathic effect in confluent monolayers, inhibition of RNA synthesis, and inhibition of cell growth in newly seeded cultures. Although this compound is not a specific inhibitor of RNA synthesis, the inhibition of RNA synthesis and the production of cytopathic effects run close together. At concentrations that indirectly affect the nucleic acid synthesis, sufficient disturbance is caused in other areas of the cellular metabolism to lead to a general cytopathic effect. Cell division is affected at lower concentrations and in this particular case, inhibitory (and therefore toxic) effects can be detected with concentrations 10 times lower than those that cause cell destruction. Nevertheless, even with cell growth as a measure of toxicity,

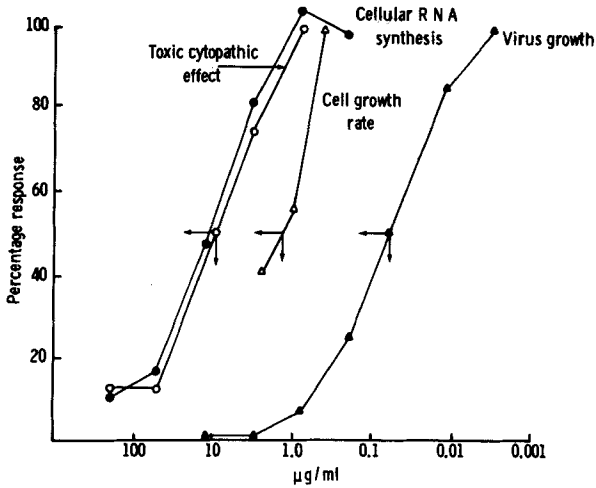


FIG. 1. Effects of ICI 65,709 on the growth of rhinovirus type 2 (▲—▲) in human diploid lung cells. The concentrations of compound required to produce toxic cytopathic effects (CPE) (○—○), effects on cellular ribonucleic acid (RNA) synthesis (●—●), and effects on the cellular growth rate (△—△) are higher than those that suppress virus growth, indicating that ICI 65,709 is exerting a specific effect on virus growth. (50% end points: CPE, 10 µg/ml; RNA synthesis, 10 µg/ml; cell growth, 1.5 µg/ml; virus growth, 0.05 µg/ml.)

there is a clear margin between the toxic and antiviral effects, as may be seen from the virus yield curve.

Fusidic acid, which has been reported to show specific antiviral activity in tissue culture (Acornley *et al.*, 1967), was also tested in the same way (Fig. 2). In this case, the concentration causing 50% cytopathic effect after 48 hours was 100 μg/ml, whereas virus yield was depressed to 50% by only 3 μg/ml, giving an apparent therapeutic ratio of 33. But when the effects of fusidic acid on cellular synthesis were studied, it was clear that cellular RNA synthesis was drastically reduced by 3 μg/ml. Thus, fusidic acid probably reduces virus growth by inhibiting cellular, rather than virus, synthetic processes, and probably accounts for the fact that this compound showed no clinically useful effects in virus-infected volunteers, despite good levels of drug in blood and nasal secretions (Acornley *et al.*, 1967).

Since the toxic effects of compounds *in vitro* usually increase with time, it is important when comparing toxicity and antiviral activity, to ensure that the compound has been in contact with cells for the same length of time in each case. In the above experiment, the cytopathic effect, RNA synthesis, cell growth, and virus inhibition were all measured in cells that had been exposed to the compound for 48 hours.

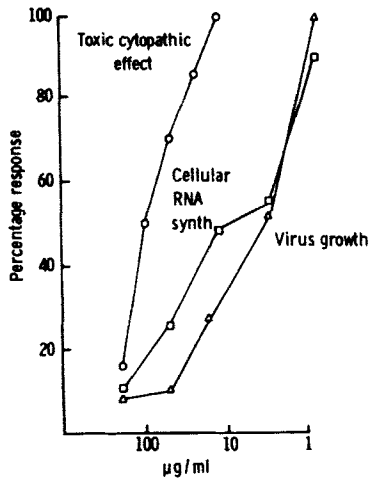


FIG. 2. Effects of fusidic acid on the growth of rhinovirus type 2 in human diploid lung cells (△—△). Similar concentrations cause a profound inhibition of cellular ribonucleic acid (RNA) synthesis (□—□), although higher concentrations are needed to cause toxic cytopathic effects (CPE) (○—○). (50% end points: CPE, 100 μg/ml; RNA synth., 6 μ/ml; virus growth, 3 μg/ml.)

### 3. *Organ Cultures*

When a virus disease is limited to a particular target organ, such as the respiratory tract, it is of great value to be able to culture a portion of the organ for *in vitro* studies. The culture of portions of trachea has been extensively used to study the growth of respiratory viruses (Hoorn and Tyrell, 1965, 1966; McIntosh *et al.*, 1967; Craighead and Brennan, 1968; Herbst-Laier, 1970), and recently organ cultures of human embryonic gut have been used to study the agents of human "virus" gastroenteritis (Dolin *et al.*, 1970). The technique could presumably be extended to the study of viruses, such as polio, rabies, smallpox, and herpes, which localize in specific organs of infected individuals.

We have found the use of human embryo and animal tracheal pieces of value in studying the toxicity and the antiviral activity of leads produced by tissue culture screening programs. Our technique is to excise a trachea and cut it transversely into rings 1–2 mm thick. These are placed in 3 × $\frac{1}{2}$ in. tubes with 1 ml of Eagle's medium and rolled exactly as conventional tissue cultures. With a low-power microscope the ciliary activity of the respiratory epithelium is assessed on an arbitrary scale of 0 to 4. In the presence of a test compound, the reduction of ciliary action is a highly sensitive measure of compound toxicity, and it is easy to determine the concentration at which full ciliary activity can be maintained (Fig. 3). At lower concentrations the effects on virus growth may be measured by harvesting the culture fluid at intervals and titrating for infectious virus. By using this technique, we have found that almost 70% of the so-called active compounds produced by a tissue culture screen against influenza A appear negative when tested in ferret tracheal cultures. In almost all cases, compounds were toxic at lower concentrations in tracheal cultures than in conventional tissue culture monolayers, as judged by a cessation of ciliary activity. In a proportion of these compounds, some data concerning their biochemical action were available, and in most cases these drugs were uncouplers of oxidative phosphorylation, nucleic acid inhibitors, or general antimetabolites. The inhibition of ciliary action in tracheal cultures is, therefore, a much more sensitive index of toxic effects than morphological changes in monolayers.

In Fig. 3, the 50% cilia-inhibitory concentration of ICI 65,709 is 2.0 μg/ml. This effect is detected at a concentration 5 times lower than is necessary to cause morphological changes in conventional tissue culture cells (Fig. 1), presumably because the metabolic patterns of the ciliated cells are more complex than those of static cells in culture and are, therefore, more readily disturbed.

In general, the concentrations of compounds that suppress ciliary activity

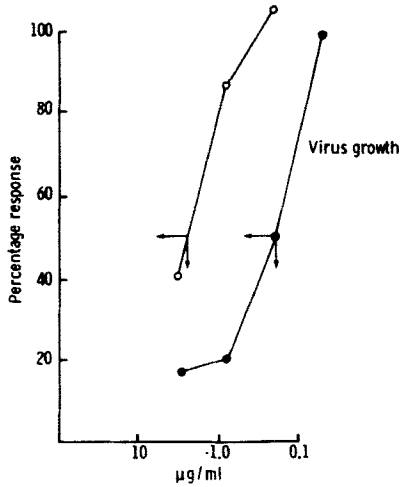


FIG. 3. Effects of ICI 65,709 on the growth of rhinovirus type 2 in fragments of human embryonic trachea (●—●). Higher concentrations are required to inhibit ciliary activity in tracheal fragments (○—○), indicating that the compound is exerting a specific effect on virus growth. (50% end points: ciliary activity, 2.0 μg/ml; virus growth, 0.2 μg/ml.)

are comparable to those that prevent cell growth, except in the case of specific inhibitors of DNA synthesis for which cell division and growth are usually more sensitive than ciliary activity.

4. *Virus Growth*

Any conventional technique may be used to measure virus growth in antiviral tests—cytopathic effect, plaque reduction, yield of infectious virus, and hemadsorption, being the most common. Because of their simplicity, cytopathic effect and hemadsorption are widely used, but these techniques must be used with some precautions if certain types of antiviral activity are not to be missed. In order to speed up the rate of antiviral testing, the quantity of challenge virus is often increased to a theoretical maximum of 1 virus particle per cell. The whole cell culture then behaves synchronously, and results are obtained in whatever time the virus takes to complete its replicative cycle—usually between 10 and 20 hours. With this procedure, however, a test compound that prevented the formation of infectious virus but was unable to protect the infected cell from destruction would not be detected. For example, in a relatively complex virus, such as influenza, it is not difficult to imagine that RNA synthesis

could be interrupted while hemagglutinin production continued, much as it does in the Van Magnus effect. The result would be a monolayer showing full hemadsorption and yet no transmissible virus would have been formed. Test compounds that produce this effect would be of great interest as potential drugs but could be missed in tests where the dose of challenge virus is too high. Wherever possible, tests should permit several cycles of virus growth to occur so that compounds that interrupt any part of the cycle may be detected.

### B. Animal Screens

There is a school of thought that tissue culture testing is so artificial as to be of little value in detecting useful antiviral substances. It is argued that by testing for antiviral effects directly in animals, the activity that is detected is likely to be more valid and more useful than that detected in tissue culture. It is true that the majority of active compounds detected in tissue culture screens are not active in animals, even after full authentication of the antiviral activity by tests such as those discussed above. The reason for this is usually that the compounds do not reach the target organs in sufficient amounts to show activity rather than because of some intrinsic defects in the antiviral activity of the compounds. Also, apart from interferon inducers, unless a compound manifests some activity *in vitro*, it is highly unlikely to do so *in vivo*.

On the one hand, it is argued that a virus growing in a tissue culture cell is of little relevance to the processes by which that virus causes disease in the whole animal. On the other hand, it can be said that, since virus growth is the basis of the pathological processes, if virus growth can be halted, then the disease can be stopped. Furthermore, the literature discloses that some highly irrelevant viruses are being used in animal test systems and that, even when human pathogens are used, e.g., influenza, they require extensive adaption to their animal host and the course of the disease is usually very different from that in humans. Finally, there are no convenient animal models for studying the growth of human rhino- and coronaviruses and, unless tissue culture tests are used, there could be no screening for antiviral compounds against these important pathogens.

There is, therefore, no convincing theoretical advantage in using animals for routine antiviral screens. This, together with the cumbersome nature of animal tests and the difficulties of "scaling-up" to test large numbers of compounds makes tissue culture testing a more attractive proposition for the initial screening program.

The foregoing remarks apply, of course, only to the detection of compounds that have a direct effect on specific virus processes, e.g., absorption,

penetration, and replication. In the field of interferon inducers, immune enhancers, and other stimulators of the host defense mechanisms, obviously one has no choice but to use test animals; but here one is concerned to detect any overall effect on the course of a disease rather than accurately to model a particular human or veterinary infection. Accordingly, the choice of test system is less critical provided it fulfills certain requirements. For instance, (*a*) the virus and test compound should be administered at separate sites to avoid any possible local destruction of the challenge virus by test compound; (*b*) the test compound should be given parenterally to give the best chance of absorption, and (*c*) the dose of challenge virus should be sufficiently small to allow a useful incubation period before symptoms develop.

The following system has been used successfully by us. Groups of 5 mice are dosed intraperitoneally with test compounds at 50, 12.5, and 3.1 mg/kg on four successive days. Twenty-four hours after the first dose, they are challenged intramuscularly with 100 $MLD_{50}$ of Semliki Forest virus, and after the last dose they are observed for symptoms twice daily. The mean reciprocal day of death (MRDD) for each test group is calculated after 14 days and compared with that of a similar undosed group as well as with that of a group given four daily injections of a protective agent such as polyinsinic-polycytidilic acid (poly IC). Figure 4 shows the typical response of mice treated with poly IC and untreated controls. Under these particular

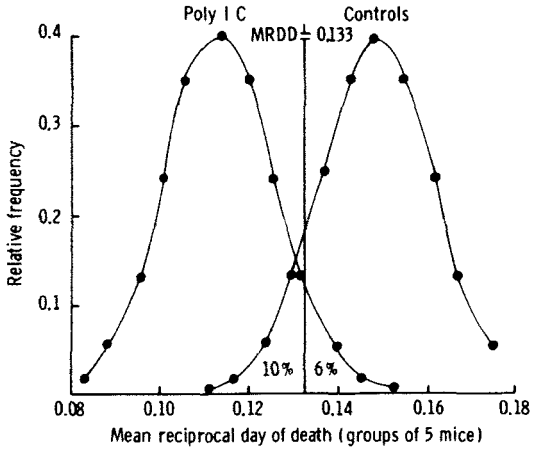


FIG. 4. Distribution of deaths, as mean reciprocal day of death (MRDD), in groups of 5 mice infected with Semliki Forest virus. The protective effects of polyinosinic–polycytidilic acid (poly IC) are shown by the displacement of the distribution curve to the left. (See text for explanation of 6% and 10%.)

conditions, the two response curves overlap. By selecting an MRDD of 0.133 as the criterion of "active" or "inactive," then theoretically 10% of all inactive compounds tested will appear as spurious actives and would require to be retested to establish their true status. Some caution is needed in interpreting the converse overlap. Given 100 known active compounds, or a single active compound tested 100 times, then 6 tests in every 100 would miss such a compound. But in practice, the vast majority of compounds passing through the test will be inactive, and the chance that the occasional true active compound will by chance fall into the "6% missed" category is correspondingly reduced.

Even with an *in vivo* test reduced to such a minimum as this, it still requires a large effort in terms of manpower and facilities to test realistic numbers of compounds.

## IV. Animal Models

Although many human viruses will grow in animal hosts it is often difficult to assess the potential value of an antiviral compound for human use by using animal models. There are five main reasons for this. First, a relatively benign human virus infection will often follow a very different, and often severe course in an animal. For example, influenza virus, herpes simplex, and coxsackie viruses may cause much more serious diseases in laboratory animals than they do in man. Second, human viruses often must be adapted by multiple passaging before they will grow satisfactorily in animal hosts, and, therefore, the challenge virus in the animal model may be a very different creature from the original human pathogen. Third, the quantity of virus administered to an animal in order to produce some measurable effect, e.g., symptoms, virus isolation, and seroconversion, is usually vastly greater than would ordinarily be encountered by the natural host, and this can have a profound bearing on the efficacy of any curative agent. For example, Finter (1967) has shown that the protection offered to mice by doses of interferon is greatly increased as the quantity of challenge virus is reduced. Fourth, the fate of a drug when administered to an animal may be very different from that seen in man. And last, a compound may show toxic effects in man which it did not show in animals. The last two points are probably the most important in determining how far the results obtained with animal models are relevant to man.

In theory the above considerations should operate in both directions; that is, a compound that shows a positive result in an animal model may be positive or negative in man, and a compound that is negative in an animal may be negative or positive in man. But, since many animal models offer a

greater challenge to the therapeutic potential of a drug than the natural disease in man, a positive result in an animal model always gives great hopes that a positive result might be achieved in man. This consideration often encourages the evaluation of potential antiviral drugs in man on the very slimmest of grounds.

An example of the dilemma that an animal model may pose is afforded by the work of Boyle and his colleagues (1970) on the compound SKF 30097. This compound was shown to be active against a number of viruses, including a wide range of human rhinoviruses, in tissue culture. The problem arose of how to evaluate this compound *in vivo*. There are no small-animal models of human rhinovirus infections, and the authors, therefore, decided to test the compound in chimpanzees, which are one of the few primates susceptible to human rhinoviruses. Because of lack of knowledge on infectivity of human rhinoviruses for chimpanzees, the authors gave up to 10,000 $TCD_{50}$ of challenge virus to each animal to ensure infection. The animals were given the drug orally 3 times a day, and the course of the disease was monitored by virus shedding from the nose. The rate of antibody rise was also measured. The numbers of animals in each experiment were necessarily small—usually 2 or 3 treated with drug and 2 or 3 controls. The final results were tantalizingly inconclusive: not clearly negative, nor convincingly positive that the drug had produced a curative effect. The investigators admit that this system is far from satisfactory but conclude from their results that the compound is worthwhile studying further in human subjects. The same conclusion, however, would probably have been reached if the compound had been clearly inactive in the chimpanzees, on the grounds that the excessive doses of challenge virus and unknown factors in the chimpanzee metabolism could have led to this result.

Animal models of human virus disease must at best be regarded as poor imitations of the natural condition, and results obtained with animal models, whether they are positive or negative, encouraging or discouraging, should be interpreted cautiously and never be used as the sole basis for predicting the outcome in man.

## V. Prospects for Antiviral Drugs

Research into the treatment of virus diseases by drugs is at present directed toward three general areas: (*1*) attempts to stimulate the defense mechanism of the host animal; (*2*) large screening programs to find drugs that directly block some virus-specific process; and (*3*) alleviation of the symptoms of the disease.

The first approach is exemplified by the variety of interferon inducers which are at present under intensive study. A disappointing feature of these is their uniformly low activity in man, despite highly promising results in laboratory animals, such as mice, rats, and rabbits. Perhaps the interferon response in man and primates is less important in defense against virus disease than it is in other taxonomic groups. Searches are being made for more general stimulants of host defense mechanisms, for example, stimulators of phagocytosis and the immune response, but very little progress has been reported so far.

The search for drugs that will directly inhibit virus replication, by stopping a virus-coded synthetic process, or the absorption, penetration, uncoating, assembly, or release of virions, has been intensive and is still continuing. Nevertheless, the products of this effort will find application only in a relatively small number of virus diseases for the reasons outlined above. Only for those diseases of high economic importance, and for which no effective vaccines are available, will specific antiviral drugs ever be a commercial reality. The present paucity of such drugs is undoubtedly due largely to the intimate association of viruses at the molecular level with their host cells. For this reason, disease targets must be carefully defined, screening procedures made as meaningful as possible, and the limitations of animal models be clearly recognized. Only by attention to these details can the maximum effort be brought to this difficult problem. Again, the lack of clinically useful drugs allows no more than speculation on the possibilities of drug-resistant viruses emerging when antiviral drugs are eventually in widespread use. The phenomenon of drug resistance in viruses is well established in the laboratory (Melnick *et al.*, 1961; Tamm and Eggers, 1962; Renis and Buthala, 1965), and, unless potential antiviral drugs are free of this serious defect, their commercial life will be embarrassingly short.

The treatment of the symptoms, rather than the cause of a disease, has been the mainstay of medical practice from time immemorial, and this is still the case with most virus disease. The short incubation period of many virus diseases will inevitably restrict the therapeutic use of antiviral drugs, and in cases where symptoms have already appeared, the physician and layman alike will have recourse to the extensive armamentarium of palliatives available for alleviating the symptoms of virus disease. All the same, this is clearly an unsatisfactory state of affairs, and the ultimate goal of antiviral research is the prevention of virus disease. As the understanding of viruses increases, so a more rational approach to the chemotherapy of virus diseases will become feasible. Also, random discoveries of antiviral activity in novel chemical compounds will shed further light on those areas of virus metabolism that are susceptible to chemical attack. With increasing con-

tributions from both these approaches, virus chemotherapy should soon emerge from a theoretical possibility to a practical reality, and antiviral drugs will make their long awaited contributions to clinical medicine.

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