REVIEW ARTICLE

APPROACHES TO THE CHEMOTHERAPY OF VIRUS DISEASES

By E. Weston Hurst, M.D., D.Sc., F.R.C.P.

Head of the Sub-Division of Micro-organismal Research, Biological Department, Imperial Chemical (Pharmaceuticals) Ltd., Manchester

Antibacterial Drugs and the Chemotherapy of Infections Caused by the Largest Viruses

Many years ago, Ehrlich laid what are now generally recognised as the foundations of chemotherapy, but before 1935 positive achievements were, by modern standards, relatively modest. Syphilis could be treated slowly with the arsenicals; trypanosomiasis had responded partially to the efforts of the synthetic chemist; and malaria was controlled by that gift of nature, quinine, while plasmoquin and mepacrine were recent therapeutic introductions. Domagk's discovery of the sulphonamides radically altered the situation. Even more important than their intrinsic therapeutic value, was their effect in directing chemical effort and research into the new and hitherto inadequately explored channel. When, a year or two later, Florey was able to develop an interesting laboratory phenomenon with *Penicillium notatum* into an outstandingly active therapeutic remedy of the lowest conceivable general toxicity, he instigated an additional vast amount of research into chemotherapeutic substances produced by living organisms. The result was an impressive degree of control over many bacterial infections achieved in a total period of little more than a single decade.

The chemotherapy of virus diseases is a more recalcitrant problem. With the exception of the largest viruses of the psittacosis-lymphogranuloma group, which some workers no longer recognise as true viruses, no practical means of influencing virus diseases by chemotherapy yet exists. This is not perhaps unexpected when we reflect on the differences between a typical bacterium and one of the smaller viruses. The bacterium is at all stages a self-contained system both morphologically and biochemically. It is always recognisable with the microscope as a discrete independent organism. Biochemically it is provided with a complicated series of enzymes which convert the nutrients it encounters into bacterial protoplasm. It is true that usually these nutrients must consist of relatively simple molecules provided by the terrestial environment or by the infected host, but within these limits bacteria possess more or less complete autonomy and retain completely their individuality. When infecting a mammalian host, many of them grow extracellularly in the tissue fluids.

On the other hand, viruses exhibit a much greater degree of dependence on the infected host. All are obligatory intracellular parasites and many of them are apparently devoid of the enzymatic complement necessary to carry out even the most simple metabolic processes. There is even evidence to suggest that at one stage in their replication some of them merge so completely with the substance of the infected cell as to be

undetectable by any means known to us. As Sir Patrick Laidlaw used to say, viruses "lead a borrowed life". This very intimate degree of parasitism would make it not wholly unreasonable to suppose that the future chemotherapy of virus diseases may be influenced in no slight degree by constitutional factors, nutritional or hormonal, in the host; indeed, as far as the experimental animal is concerned, this is more than mere conjecture.

The health of the animal may enter into intended chemotherapeutic experiments in a rather different context. It has long been accepted that the state of well-being or otherwise of an animal may modify its response to infection with a virus. A diseased or emaciated rabbit gives a poor skin reaction when infected intradermally with vaccinia or infectious myxomatosis. The event is no different if the sickly condition of the rabbit has been brought about by administering one of the more toxic products of the synthetic chemist, and more than one of the alleged antiviral effects recorded against a smaller virus probably comes into this category. In attempted chemotherapeutic experiments we must ensure that dosage of toxic chemical substances is within reasonable limits, and insufficient materially to affect the vitality of the animal, if we wish to avoid false positive results.

Nearly all the clinically recognised antibacterial drugs can be shown to be bacteriostatic or bactericidal *in vitro*. This is not the expression of any fundamental principle; rather does it reflect the manner in which the substances in question have been discovered. In the search for new antibacterials, the biologist has usually examined substances by determining their action on bacterial cultures *in vitro*, and has then tested the more highly active against diseases produced by these bacteria in some suitable experimental animal such as the mouse. Many substances active under *in vitro* conditions fail to surmount subsequent hurdles. Given orally, they may be destroyed in the alimentary canal or fail to be absorbed from it. After injection, they may cause such severe local irritation as obviously to be quite unacceptable in clinical practice. They may be too rapidly metabolised to attain effective concentrations in the tissue fluids at the site of bacterial multiplication, or they may be too generally toxic to be given in adequate amount. If the infecting organism is located in the central nervous system or in certain other sites, the drug may not pass the blood-brain or similar barrier, at least until infection is well established and a severe inflammatory reaction has altered the permeability of the barrier; normally substances of a definitely acidic character, e.g., penicillin, do not pass the blood-brain barrier. Thus relatively few of the substances active *in vitro* possess all the qualifications for systemic antibacterial action. To be useful in virus diseases, where so intimate a relation exists between the virus and the metabolic processes of the host-cell, a substance would need to possess still other properties. It would need to penetrate and to act intracellularly, and in such a manner as not seriously to interfere with the essential metabolic functions of the cells—unless it were able to render the cell insusceptible to infection, or to the effect of growth within it of the virus, both of which occurrences are theoretical possibilities.

Were the mouse test rather than an *in vitro* test the initial one in the search for new antibacterial drugs, it is possible that new remedies might be discovered having little or no *in vitro* activity. Indeed, it is a fact that the initial sulphonamide, prontosil rubrum, would never have been discovered by an *in vitro* test because it is not active as such but only after the sulphonamide moiety has been released by metabolism in the animal body. The number of substances showing *in vitro* activity against tubercle bacilli is very great, but few affect the disease in the mouse, so that with this infection it is usual nowadays to proceed directly to the mouse test in the search for new antituberculous remedies. Experimentally at least, three classes of substances are known to possess substantial antituberculous activity in the mouse with no or with very low activity *in vitro*: these are (i) a purine analogue, 7438[1], (ii) various surface-active polyoxyethylene ethers[2], and (iii) certain organic sulphur compounds derived from ethyl mercaptan[3]. None of these substances would have attracted notice as an antituberculous remedy by its activity *in vitro*.

Inactivation of Viruses in vitro

With viruses we find even less evidence of a correlation of *in vitro* and *in vivo* effects; indeed, the evidence available suggests that little or none exists. This fact is not perhaps surprising. Many antibacterial drugs act on the susceptible bacteria when these are in some particular phase of their cycle of growth: outside the living body viruses are in a state of suspended animation and perhaps only susceptible to crude chemical influences which grossly disrupt their substance. Thus *in vitro* they are inactivated by acids and alkalies, by oxidising agents such as hydrogen peroxide, potassium permanganate, hypochlorite or even atmospheric oxygen, by general protoplasmic poisons, such as phenol, mercuric chloride and formalin, and by some surface-active agents, such as sodium lauryl sulphate, bile salts and saponin. Many of these substances act efficiently only at relatively high concentrations, but iodine and other halogens are remarkably destructive to the influenza virus[4] and inactivate it almost instantaneously at an atmospheric concentration of around one in ten million, provided that the virus is in the form of a wet mist; if the virus is dried rather higher concentrations of iodine and longer exposure are necessary. Similarly, viruses are susceptible to a variety of physical influences such as ultra-violet and X-ray irradiation, ultrapressure, etc. None of the chemical substances mentioned, nor a great number of others capable of inactivating viruses *in vitro* has any action against virus diseases in the experimental animal. However, the chemical or physical inactivation of viruses *in vitro* is of some interest in two connections which have nothing directly to do with chemotherapy.

One point concerns the disinfection of contaminated material. Some years ago we examined a number of modern surface-active agents and antibacterial substances such as the quaternary ammonium compounds and chlorhexidine (Hibitane) for power to inactivate viruses. To one of the fairly large viruses, that of herpes febrilis, an anionic detergent,

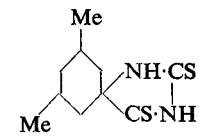
sodium cetyl-oleyl sulphate, was moderately destructive; a non-ionic detergent and the other substances were without effect. None of the substances tested inactivated two of the smallest viruses, those of mouse poliomyelitis and Rift Valley fever, at concentrations which would be at all economic to use in practice. Sodium hypochlorite, on the other hand, was exceedingly potent in this respect and superior in every way to the other compounds examined. Ultra-violet light and some chemical substances mentioned below have been used to destroy in transfusion plasma or blood the agent of infectious jaundice, with somewhat inconsistent results. The virus is apparently one of the more resistant ones, and one of the difficulties is to give an adequate exposure to the inactivating agent without causing undesirable chemical changes in the serum proteins[5–7].

Secondly, and of some topical interest at the present moment, is the use of chemical inactivating agents for the production of viral vaccines. In the past, substances such as phenol and formaldehyde have been employed almost exclusively for this purpose. They suffer from the great disadvantage of being relatively non-specific in their action and of seriously damaging all the viral proteins, including the antigenic groupings needed to stimulate antibody formation. The multiplication of viruses depends on their nucleoprotein component, and the fact that substances like phenol and formaldehyde have been used with undoubted partial success, and have apparently inactivated virus without destroying all antigenicity, may have resulted from the relatively greater lability of nucleoproteins. However this may be, it has long been recognised that to use formalin successfully in the production of a viral vaccine, the inactivating concentration is rather critical. If it is too high the vaccine loses its antigenic potency; on the other hand too low a concentration, permitting survival of living virus, presents obvious dangers. For many years discussion continued on whether a completely killed viral vaccine was, in fact, able to excite immunity, and recent American experience with the Salk poliomyelitis vaccine has underlined the great care that must be taken if disastrous events are not to follow vaccination with a formalinised vaccine. In short, it would seem that the use of formaldehyde for vaccine production is far from ideal. Only recently, however, have attempts been made to utilise some of the newer chemical substances which have much greater relative affinity for nucleoproteins, and which might, therefore, be expected to abolish infectivity while retaining unimpaired the antigenicity of the virus. Such substances are the nitrogen mustards, β-propiolactone and acetylethyleneimine. On chemical grounds the mustards are, in theory, less satisfactory than the others. All these substances are mutagenic agents and are carcinogenic, and in large doses produce agranulocytosis and radiometic effects, but as any unreacted material can readily be removed from the vaccine there need be no apprehension on this score. Our own recent experiments and those of workers in America[8–10], have suggested that vaccines produced by the agency of these substances are antigenically superior to formalinised vaccine, while the certainty of really killing the virus is guaranteed.

Large Viruses. Psittacosis-lymphogranuloma Group

Inactivation in vivo. So much for *in vitro* effects on viruses. From the point of view of chemotherapy, animal viruses may be divided into two groups—a smaller more or less effectively controlled by a number of existing remedies, and a larger for which, as yet, there exist only hints of ultimate therapeutic measures. The former includes the largest viruses of the psittacosis-lymphogranuloma group, the latter the majority of viruses.

The psittacosis-lymphogranuloma group of viruses includes the agents of psittacosis and ornithosis, lymphogranuloma venereum, trachoma and inclusion blennorrhoea together with a rather larger number of viruses naturally infecting laboratory or domestic animals. From 1938 onwards it has been known that the sulphonamides are of benefit in human cases of lymphogranuloma. Experimentally quite a number of other synthetic chemicals substances, e.g., the nitroacridines and the quinoxaline -1:4-oxides, have been found to show activity, but as one by one they have been discovered they have been completely overshadowed by successive emergence of new and better antibiotics, so that at the present moment the antibiotics hold the field. For the most part, the substances active against the largest viruses are also antibacterial, though we have knowledge of at least one substance, a spirothiohydantoin (20,065) (I), which is reasonably highly active against psittacosis in the chick embryo and mouse, yet is wholly devoid of antibacterial activity *in vitro* or *in vivo*. In a second series of chemical substances under examination at the moment, as the molecular structure is changed by addition or removal of various substituents, antibacterial and antiviral activity move in opposite directions, so that it is by no means invariable to find both activities in a single substance.



(I) 20,065 (*cis* α-form)

When the literature is consulted to find which of the numerous remedies available is the one of choice in a particular instance, there is far from complete agreement. This is partly because, like bacteria, the different viruses vary among themselves in their susceptibility to the various remedies, and with two distinct remedies are not necessarily arranged in the same order of susceptibility. Thus mouse pneumonitis responds well to the sulphonamides and poorly to the nitroacridines, while feline pneumonitis, meningopneumonitis and psittacosis viruses do exactly the opposite. Lymphogranuloma responds both to the sulphonamides and to some nitro compounds. Sulphonamides act on two American strains, 6BC and Gleason, of psittacosis virus, and on rare strains met with elsewhere[11], but they are useless against most strains of psittacosis isolated in America, Europe or Australia. A second difficulty arises in assessing the value of treatment in human disease, and especially in a disease such as trachoma in which the individual severity varies greatly in different parts of the world and there is no convenient animal for laboratory study. Finally, no fully comprehensive comparison of the whole range of modern antibiotics has been undertaken at one time and by the same techniques against the viruses pathogenic for man which can easily be worked with

in the experimental animal. The advantages and the limitations experienced by the experimentalist in this kind of study are best illustrated by presenting some of the results we have obtained with the virus of psittacosis.

Psittacosis Virus. Chick Embryo and Mouse Experiments

The data on eggs were obtained in three different experiments in which the virus was injected into the yolk-sac of groups of 12 or more 7-day old embryos and followed $2\frac{1}{2}$ hours later by antibiotics. The results of three experiments were virtually identical and have been combined in Table I;

TABLE I

THERAPY OF PSITTACOSIS IN THE CHICK EMBRYO

Antibiotic	Dose mg./egg	Per cent survivors	Mean period of survival in days*
Tetracycline (Achromycin)	5·0	79·2	13·5
	1·0	91·7	13·8
	0·2	79·2	13·5
	0·04	41·7	10·6
	0·008	0	7·6
Oxytetracycline (Terramycin)	5·0	75·0	13·9
	1·0	75·0	13·2
	0·2	83·3	13·3
	0·04	66·7	13·1
	0·008	0	6·6
Chlortetracycline (Aureomycin)	5·0	83·3	13·4
	1·0	79·2	13·5
	0·2	0	7·9
	0·04	0	5·5
Erythromycin (Ilotycin)	5·0	37·5	12·2
	1·0	91·7	13·7
	0·2	91·7	13·9
	0·04	33·3	12·3
	0·008	0	5·9
Penicillin	1·0	20·8	12·8
	0·2	0	10·1
	0·04	0	7·3
Chloramphenicol (Chloromycetin)	2·5	4·2	7·6
	1·0	0	9·3
	0·2	0	5·6
	0·04	0	5·0
Carbomycin (Magnamycin)	10·0	20·8	7·6
	2·0	41·7	9·4
	0·4	0	6·2
	0·08	0	5·7
None	—	0	5·0

* Eggs surviving to the time of hatching (13th day after virus) were deemed to have survived for one additional day.

they showed that, in treating yolk-sac infections with the virus of psittacosis, chlortetracycline (aureomycin) is definitely inferior to the other two tetracyclines, as we found also on a previous occasion[12]. The same has been reported for other organisms of the group[13,14]. Erythromycin is equally as good as the better tetracyclines, sodium penicillin and carbomycin are much less effective with about the same degree of activity, and chloramphenicol is relatively ineffective. The best results are not always obtained with the largest dose, a fact attributable to toxicity from excess of the antibiotic.

Four experiments were made in groups of 10 or more mice infected intraperitoneally with psittacosis (Table II). After inoculation the animals remained untreated for 48 hours to allow the infection to become established and were then dosed orally twice daily for 10 days, except with procaine-penicillin where a massive dose of the antibiotic was given subcutaneously once every third day, beginning 48 hours after virus. As

TABLE II

THERAPY OF PSITTACOSIS IN THE MOUSE

Antibiotic	Dose mg./20 g. twice daily	Per cent survivors
Tetracycline (Achromycin)	10·0	100
	1·0	100
	0·1	98 (7·4)†
	0·01	13 (7·4)†
	0·001	5 (6·7)†
Oxytetracycline (Terramycin)	10·0*	100
	1·0	100
	0·1	100†
	0·01	13 (7·6)†
	0·001	0 (6·1)
Chlortetracycline (Aureomycin)	10·0*	100
	1·0	100
	0·1	100
	0·01	95 (7·5)†
	0·001	18 (6·4)†
Erythromycin (Ilotycin)	10·0	100
	2·0	100
	0·4	93 (12·5)†
	0·1	10 (7·1)†
Procaine-penicillin	See text	100
Chloramphenicol (Chloromycetin)	10·0	100†
	2·0	90 (18·0)†
	0·4	65 (13·8)†
	0·1	3 (6·2)†
Carbomycin (Magnamycin)	10·0	100
	2·0	45 (11·2)†
	0·4	—
	0·1	5 (6·8)†
None	—	3 (6·2)†

The figures in parentheses are the mean periods of survival in days of mice ultimately dying.
* Signs of toxicity of the drug.
† Surviving mice had shown signs of infection.

we found in a similar experiment some years ago, in the mouse, chlortetracycline is the most active of the tetracyclines, thus reversing the position in the chick embryo and showing how difficult it is from work in one host to predict the effect in another. If, in spite of this, one were pressed to predict activity for man, our cumulative experience over the years would suggest that it is better to rely upon the results in the mouse rather than those in the egg. In the mouse, erythromycin also is relatively less active than in the egg and is very much inferior to chlortetracycline. Chloramphenicol and carbomycin are still less active. A massive dose of procaine-penicillin given as mentioned above will protect 100 per cent of the mice, but the doses used (30,000 units/mouse) are enormous relative to those employed in man, and smaller doses are not nearly as effective. Penicillin is also useless if virus is given intracerebrally because it does not

penetrate the blood-brain barrier, whereas under these conditions the tetracyclines are still highly active. To place these results in their fullest perspective, it should be added that against the virus used in this work, sulphadiazine or sulphamezathine possess little, if any, activity, while the most active nitroacridines have a moderate action[15–17], and quinoxaline-1:4-oxides a very considerable action[18] approaching that of the better antibiotics.

For some of the antibiotics we have prepared growth curves of the virus in treated and untreated animals. Groups of six mice were killed at various times after intraperitoneal infection, the spleens pooled and the amount of virus present estimated by titration in mice. In these experiments treatment started at 4 hours before infection with virus so as to obtain the maximum effect possible. The results indicated very considerably reduced multiplication of virus during the period of therapy in animals treated with chlortetracycline and with penicillin, whereas chloramphenicol appears only to delay virus in reaching a high titre[12].

By contrast, *in vitro* these antibiotics have seldom been found to have any effect on the viruses susceptible to their action *in vivo*; at least this holds with reasonably large doses. Using very high concentrations of penicillin, Moulder and his colleagues[19] found that the antibiotic forms a complex with the virus of feline pneumonitis with resulting loss of infectivity, but as this effect was partly reversible by treatment with penicillinase it cannot represent a true inactivation of virus.

The effect of treatment with penicillin can also be visualised in histological sections of the yolk-sac stained with Giemsa's solution or other suitable stains[18,20]. The normal colonies of tiny basophilic particles which represent the actual virus are replaced by large, deeply-staining plaques measuring up to 6 μ in diameter. In the presence of penicillin the virus particles apparently continue to grow indefinitely without subdividing as they do in the untreated egg. In the treated eggs, moreover, the number of cells infected with virus is much lower than in the untreated controls.

In spite of the excellent clinical results and the frequent failure to detect virus during the later stages of the period of therapy, none of these antibiotics certainly eradicates the virus. After treatment has ceased, virus frequently reappears and many mice carry virus for months, though they do not infect other mice placed in the same cage. In the experiments described, the spleens were removed from the treated mice and passed individually to fresh mice on the 40th day after infection, that is to say, 28 days after the close of treatment. At this time all the surviving mice looked perfectly well; nevertheless the majority carried virus (Table III). We believe that it is the immunity engendered during the period when viral growth is controlled by therapy which prevents unrestricted growth of virus after drug is discontinued. On many occasions we have noted that penicillin gives lower carrier rates than do the other antibiotics and it may be that tetracycline is less satisfactory in this respect than are chlortetracycline and oxytetracycline (terramycin).

Similar behaviour is, of course, known in man. Meyer and Eddie[21] have described a case of psittacosis in which virus was still present in the

sputum 10 years after infection, in spite of an intervening course of penicillin therapy. Recently, however, by very heavy and prolonged parenteral dosage with the tetracyclines, both Schmidt and von Sprockhoff[22] and Meyer and Eddie[23] have succeeded in freeing animals from virus. The dosage required is relatively enormous by the standards of human therapy yielding excellent clinical results, and these doses may have to be continued for up to 25 days.

TABLE III

CARRIERS OF PSITTACOSIS VIRUS AFTER TREATMENT WITH ANTIBIOTICS

Antibiotic	Dose mg./20 g. twice daily	Per cent carriers
Tetracycline (Achromycin)	10·0 1·0–0·1 <0·1	80 94 100
Oxytetracycline (Terramycin)	10·0 1·0–0·1 <0·1	60 86 100
Chlortetracycline (Aureomycin)	10·0 1·0–0·1 <0·1	50 86 100
Erythromycin (Ilotycin)	10·0–2·0 <2·0	87 93
Procaine-penicillin	See text	45
Chloramphenicol (Chloromycetin)	10·0 2·0–0·4	72 83

Regarding the possibility of drug-resistance, the available evidence is reassuring. It is true that a few resistant strains of lymphogranuloma have been isolated from patients treated with the sulphonamides and that similar resistance can be produced fairly easily experimentally. But no strains similarly made resistant to the antibiotics have been reported from the clinics, while under experimental conditions, most workers, including ourselves, have not succeeded in obtaining drug resistance in mice to penicillin or to chlortetracycline. Moulder and his colleagues[24] in the chick embryo succeeded after many passages in the presence of increasing amounts of penicillin in producing a strain of feline pneumonitis resistant to the drug, but the process does not appear to have been an easy one and it seems rather unlikely that under the conditions obtaining in human medicine, drug-resistance of the larger viruses to the antibiotics will prove a serious problem.

Status of Therapy in Man

The results of therapy in man are in fair agreement with those which have been reported in the experimental animal. As we have seen, the partial efficacy of the sulphonamides in lymphogranuloma venereum was first noted in clinical practice, and only later applied to the mouse and the chick embryo. Penicillin has been reported to be active, but apparently has seldom been used in this disease. There seems to be little doubt that the tetracyclines are the antibiotics of choice. This is undoubtedly true

also of psittacosis: against most strains of virus the sulphonamides are ineffective, and the reports of treatment with penicillin have been mixed. In the presumed virus disease or diseases passing under the name primary atypical pneumonia, therapeutic effects have been claimed with chloramphenicol or the tetracyclines after therapy with sulphadiazine or penicillin has failed. It is when we come to trachoma that opinions differ to a greater extent. By various observers, the sulphonamides have been considered ineffective in treatment, as useful adjuncts to therapy either in dealing with secondary infections or in supplementing other measures, or as curative agents in themselves. One American ophthalmologist of considerable experience[25] even maintains that they still constitute the first therapeutic choice and are demonstrably superior to the antibiotics. Penicillin certainly does not seen to have established itself in the treatment of this disease, but most recent reports suggest that once again the tetracyclines are superior to other agents, including chloramphenicol. An important limitation to their usefulness is that they are too expensive to be distributed widely in the impecunious countries in which trachoma is rampant. The extent of the need for a cheap synthetic remedy for trachoma is emphasised by facts such as the following—8 of 10 Egyptians are said to suffer from trachoma or its effects—millions of man-days are lost annually in Tunisia from trachoma—trachoma is the commonest cause of blindness in the British colonial territories and currently accounts for over 80,000 cases. Thus trachoma continues to be one of the most important targets for chemotherapy in the field of virus diseases.

Problems of the Chemotherapy of Diseases Caused by the Smaller Viruses

With diminishing size it is obvious that the viruses smaller than those of the psittacosis-lymphogranuloma group must decrease progressively in structural complexity. By implication they must be increasingly dependent on the cell which harbours them for supplying the essentials for their reproduction. Vaccinia virus, with a diameter of about 250 mμ, has been said to have a fairly complicated structure under the electron microscope, and to contain when purified various enzymes, a co-enzyme and other substances. It is not at all certain, however, whether some of these constituents may not have been acquired during or after the process of separation from the host-cell. Influenza virus (125 mμ), which for technical reasons has been most studied in this connection, is certainly a rather complicated affair. Relatively little attention has been devoted to the still smaller viruses, though all that have been purified have been shown to contain protein and nucleic acid, the latter usually ribosenucleic acid. It is generally believed that it is the nucleic acid which is essential for reproduction of the virus.

The Eclipse Phenomenon. Influenza Virus

When many of the smaller viruses infect a susceptible tissue, a rather characteristic phenomenon ensues. Within a very short time, sometimes only a few minutes, of introduction of a suitable infecting dose, infective

virus drops sharply in amount and there follows a period of so-called eclipse during which little or none of the original virus can be recovered. After a certain time virus reappears and its concentration rapidly increases. One could imagine several reasons for this disappearance of virus. For example, much of it might be adsorbed or be inactivated by the defences of the host, leaving an undetectable amount to survive and initiate infection, and indeed since the phenomenon was first discovered, opinions regarding its significance have differed widely. No-one apparently has ever published what may be an important observation—namely of the fate of a given virus introduced into sites in which it is, respectively, capable and incapable of growth. Some years ago we performed one such experiment. Using influenza virus which grows readily in the mouse lung but not in the mouse testicle, we determined the infectivity of these tissues at intervals after introduction of virus. Within a few minutes the virus introduced into the lung fell to a level below that necessary to infect other mice intranasally, that is to say it suffered the phenomenon of eclipse—that in the testis disappeared rather slowly over about 46–60 hours without nearly as marked an immediate drop in infectivity. This of course suggested that eclipse of the virus is a phenomenon intimately associated with its multiplication. Unfortunately, we have not yet had the opportunity of confirming this observation. In a stimulating if admittedly speculative article, Bauer in 1949[26] summarised the then current knowledge of viral multiplication, and concluded that on entering a susceptible cell the virus undergoes dissolution, diffuses over a considerable area of the cytoplasm, and then, by some means or other, induces the enzymes of the cell to manufacture viral material instead of normal cellular material. Subsequent observations with at least one virus have given considerable support to this general thesis.

The studies of Hoyle and others over the last few years have provided a great deal of evidence for what happens to the influenza virus during the period of eclipse. The virus of influenza possesses a number of properties by which it may be recognised. Among these are the following:

1. The infective particle has a diameter of 125 mμ, or 1250 Å. 2. The virus is capable of attaching itself to receptor sites on the surface of the red blood corpuscles of various species and of causing these red blood corpusles to become agglutinated. 3. While the virus is attached to the red blood corpuscles, an enzyme, a mucinase, which is incorporated in the viral particles, destroys the receptor substance necessary for attachment of the virus. In consequence the virus parts company with the red blood corpuscles and these cease to be agglutinated. 4. The virus contains specific and group complement-fixing antigens.

After shaking a suspension of influenza virus with ether, the virus disintegrates, according to Hoyle[27] from disruption of a lipoid-containing surface membrane derived from the host-cell which previously harboured the virus. The suspension now is no longer infective and the particles present are much smaller than the intact virus and are mainly about 120 and 250 Å in diameter. However, some of the properties of the original virus remain. The haemagglutinin titre is higher than before. The

specific complement-fixing antigen has gone, so that Hoyle concluded that it represents the part of the virus derived from the host-cell. The group antigen persists and by suitable means can be separated from the haemagglutinin. The two activities appear to be associated with separate particles. The group, or so-called "soluble" antigen is a particle 120 Å in diameter, with the properties of a ribonucleoprotein; it contains all the nucleic acid of the virus. The agglutinin behaves like a mucoprotein with enzymatic properties. Current information of the composition of the influenza virus is summarised in Table IV.

TABLE IV

COMPOSITION OF THE INFLUENZA VIRUS

INFLUENZA VIRUS 1250 Å			
"Soluble" antigen	120 Å	14 per cent	Ribonucleoprotein (M.W. 600,000) containing 5·3 per cent ribonucleic acid—70 molecules
Haemagglutinin	120 Å or more	14 per cent	Mucoprotein with 4·2 per cent polysaccharide— 70 particles
Lipid .. .. Carbohydrate .. Protein	— — —	29–36 per cent 3 per cent 34 per cent	Derived mainly from host-cell

Inside the infected cell viral material can be sought for by several techniques, all of which have been used by one or other investigator. The virus may be labelled with radioactive phosphorus[28], it may be looked for with the electron microscope, or the antigenic material may be demonstrated by the application of antibody which has been rendered fluorescent. By labelling influenza virus with radiophosphorus, Hoyle and Frisch-Niggemeyer[29,30] were able to show that on entry into a susceptible cell much the same happens to the virus as when it is shaken with ether. Almost immediately infectivity is lost, and the virus particle as such disintegrates; at this stage and for some considerable time later, the electron microscope does not reveal any particles resembling virus in the infected cells[31]. About a quarter of the total radiophosphorus in the virus used for infection is contained in the phospholipid of the viral membrane, and this fraction is now recovered in the form of compounds of low molecular weight, not precipitated with protein precipitants, and not sedimented at a centrifugal force of 100,000 g.; they appear to be metabolic products taking part in the general metabolism of the cell, and incidentally reflect the very rapid and active metabolism which occurs at the cellular level. The remainder of the radiophosphorus incorporated in the virus is contained in the ribonucleoprotein fraction, and this is now recovered partly as free nucleic acid, and partly in association with the desoxyribonucleoprotein of the nuclei of the infected cell. Fluorescent antibody techniques have shown that new viral antigen appears first in close association with the nuclei[32,33]. The suggestion is that viral ribonucleoprotein breaks down to liberate free nucleic acid, which then enters into close relation with the nuclear material of the infected cell and somehow determines a changeover from synthesis of cellular ribonucleoprotein

to viral ribonucleoprotein; the two nucleoproteins differ considerably in the relative proportions of pyrimidine and purine bases which they contain.

When virus is about to reappear in the infected cell, it is preceded by smaller particles having the properties of the soluble antigen, and rather later by haemagglutinating activity[34,35]. Only at the very end of the process do these particles become associated with a lipoprotein envelope derived from infected cell, and does an infective particle of the size and with the properties of the complete influenza virus emerge from the cell surface.

Other Small Viruses

Information about the multiplication of other small animal viruses is much less complete, and it is not certain that the mechanism of replication is precisely the same in all instances, or even that all authorities would agree with the above interpretation of the facts about the influenza virus. However, it seems very likely that in essentials the process is similar. The recent interesting work with tobacco mosaic virus of plants shows that a non-infective protein fraction consists of little hollow rods into which, under suitable conditions, the nucleic acid can slip, so to speak, to form the infective virus[36]. Other artificial and natural ribonucleotide polymers can substitute for the nucleic acid, but the particles formed are not then infective[37]. With some bacterial viruses, it seems that the protein tail is responsible for uniting the virus to the susceptible bacterium and acts as a kind of hypodermic syringe for introducing the nucleic acid which is the component essential for multiplication[38].

To summarise, present evidence suggests that the nucleic acid is the essential component of viruses by means of which they gain control over the metabolic processes of the host-cell, and induce them to synthesise more viral nucleic acid thus ensuring their own survival. This essential requirement met, the new viral particle may be composed to quite a considerable extent of more or less unchanged material derived from the host-cell.

We thus reach the point of regarding multiplication of many of the smaller viruses as an exercise in nucleic acid synthesis. Nucleic acids are polymers of high molecular weight built up from purine and pyrimidine basis like adenine, guanine, cytosine, or uracil, a sugar which is either D-ribose or 2-deoxy-D-ribose, and phosphate. The manner in which these units are associated one with another, so as to create a structure capable of ensuring its own replication, is not yet certain—a convenient review of nucleoproteins is that of Markham and Smith[39]. However, our present knowledge falls far short of that needed intelligently to intervene in the process as it occurs in the mammalian host. The absence of this knowledge has not deterred workers from following certain lines of investigation in the hope of obtaining chemotherapeutic leads.

Chemotherapeutic Approach with Metabolic Analogues

Following a current trend in chemotherapy, various workers have sought to influence the course of events by the use of analogues of substances essential for cellular metabolism. In plants, with bacteriophages

and in animal tissue-cultures, the growth of viruses has been influenced by concentrations of chemical substances well below those seriously toxic to the cells. Thus the guanine analogue 8-azoguanine may modify infection with certain viruses of plants; it is actually incorporated into the viral nucleoprotein, production of virus is delayed, and the analogue-containing virus is less highly infective than is normal virus[40]. 2-Thiouracil acts in other virus diseases of plants. 2:6-Diaminopurine inhibits the growth of vaccinia and other viruses in animal tissue-cultures. Amino acids and their analogues have been examined in great variety. Methionine has been shown to be essential for the growth of several animal viruses, the multiplication of which may be inhibited by ethionine. Certain α-aminosulphonic acids were found to act exclusively on the earlier stages of multiplication of influenza virus, whereas DL-methoxinine affects a later stage just before the appearance of mature virus[41]. Analogues of vitamins have similarly been studied. Oxythiamine and desoxypyridoxine inhibit the growth of mumps and influenza viruses in tissue-culture. Tamm and his colleagues[42–44] have examined many derivatives of benzimidazole, which is a fragment of the vitamin B_{12} molecule, and by appropriate synthesis have increased their activity to a point several hundred times removed from that of the starting material. Both the 5:6-dichloro-ribofuranosylbenzimidazole and the 4:5:6-trichloro compound possess marked antiviral activity in tissue-culture. These are but a few selections from a much larger number of similar observations.

While studies such as these are providing valuable basic information on the requirements for viral growth in tissue-culture, none of the substances examined so far appears to have any useful activity in the experimental animal, and so to be of practical value as far as chemotherapy is concerned. Indeed, even results obtained in the chick embryo are unreliable from this point of view. Thus, my former colleague, Mr. J. Francis, examined many hundreds of compounds for activity against the virus of fowl-pox by injecting them into the yolk-sac of chick embryos which had received virus on the chorio-allantoic membrane. He found that a little more than a score possessed significant activity in reducing the lesions of the disease. When tested against fowl-pox in the hatched bird, however, none of these produced the slightest effect. In our own experience the only frequent correlation of results in the chick embryo and in the mouse has been with the largest viruses of the psittacosis-lymphogranuloma group; even here the correlation is not invariable and results produced in the egg may fail to be repeated in the mouse. In consequence, some workers have felt that the quickest route to chemotherapy may be the empirical screening of chemical substances directly in the mouse or other small experimental animal. Several small series of results have been published, dealing in all with a few hundred compounds, but it is virtually certain that the total examined in one or other commercial laboratory amounts to many thousands. As far as I am aware, these efforts also have been wholely unsuccessful in leading to the development of a practical remedy. Nevertheless, in the course of investigations devoted to other ends, one or two antiviral effects have been noted in the

experimental animal, suggesting that the problem of influencing diseases caused by the smallest viruses is not completely insoluble. We may perhaps consider a few of the more marked of these effects.

Other Observed Antiviral Chemotherapeutic Substances in Animals

Horsfall and McCarty[45] and Ginsberg and Horsfall[46,47] observed that polysaccharides derived from various bacterial species, and notably a capsular polysaccharide of Friedländer's bacillus, type B, are capable of lessening the severity of lesions and of restricting viral growth in infections with the pneumonia virus of mice. The polysaccharide is effective when given intranasally during the first two-thirds of the cycle of growth of the virus, that is to say, within the first ten hours, but not when given at twelve hours or later. However, the compound given late may inhibit the second or subsequent cycles of growth, so that a single intranasal dose of 0·02 mg. given two or three days after infection permits the animal to recover completely when all the untreated controls die. Immune serum given at a similarly late stage fails to save the animal. For this reason the polysaccharide was considered to act not on the mature viral particle but on some relatively late stage of viral synthesis by the host cell. The polysaccharide was completely inert against influenza viruses in the mouse, although these viruses are believed to grow in the same cells and produce apparently identical lesions as does the pneumonia virus of mice. This fact was taken as indication of different metabolic paths to the formation of the two types of virus.

Secondly, there are the thiosemicarbazones and phenoxythiouracils. A number of American workers[48–50] have demonstrated their activity against vaccinial infections in mice, but not against several other viruses. The compounds are not active against vaccinia in the rabbit. Some thiosemicarbazones, of course, are active in tuberculosis. When a range of these substances was examined, the antiviral activity was found not to run parallel with the antituberculosis. Working independently, Bauer[51] has reported on many of these substances and considers that isatin thiosemicarbazone is much more active than previously claimed. He found that in the presence of the drug the amount of virus needed to kill 50 per cent of the mice was four or more log units greater than in untreated controls: this he describes as a 99·99 per cent protection. 5-(2:4-Dichlorophenoxy)thiouracil is less active but acts synergically with the thiosemicarbazone, as do also some other phenoxypyrimidines even when themselves devoid of appreciable antiviral activity. We should note that these treatments do not by any means prevent the growth of virus in the mice, but merely restrict it to a level insufficient to próduce clinical symptoms and death. The degree of multiplication needed to produce symptoms is considerable, and when the titres reached by virus in treated animals are taken as the criterion of antiviral activity, the results are perhaps less impressive than the figures for mortality would suggest.

The third example is of an antibiotic, helenine, which was isolated by Shope[52] from a culture of *Penicillium funiculosum*, and of an apparently similar substance described by Powell and his colleagues[53]. Given near

the time of infection, these substances prolong survival or decrease mortality in infections with a number of neurotropic viruses, including poliomyelitis in the monkey[54,55].

Antiviral Action of Mepacrine

Fourthly there is the antiviral action of mepacrine. Following a line of investigation which began with the observation in America of slight antiviral activity on the part of trypan red[56], we observed that mepacrine, the antimalarial drug belonging to the chemical class of acridines, possesses very marked protective activity against a few viruses, of which equine encephalomyelitis, Rift Valley fever and louping-ill are the chief. It has no action against the majority of viruses[57,58]. It will be sufficient here to mention the results obtained in one infection—equine encephalomyelitis.

TABLE V

ANTIVIRAL EFFECT OF MEPACRINE IN EQUINE ENCAPHALOMYELITIS

A single oral dose of 10 mg. was given at various times relative to the infecting dose of virus

Time	Deaths in 20 mice
48 hr. before virus	4 (9·0)
24 hr. " "	2 (11·1)
4 hr. " "	1 (12·0)
4 hr. after virus	6 (7·3)
24 hr. " "	11 (5·3)
48 hr. " "	16 (4·6)
No drug	19 (4·7)

TABLE VI

ANTIVIRAL EFFECT OF MEPACRINE IN EQUINE ENCEPHALOMYELITIS

A single oral dose of 10 mg. was given 24 hours before various doses of virus

	Deaths in 20 mice	
Virus	Treated	Control
10^{-3}	2 (6·0)	19 (4·4)
10^{-4}	2 (11·3)	20 (4·6)
10^{-5}	3 (14·7)	16 (4·6)
10^{-6}	0	6 (10·3)

The figures in parentheses are the mean periods of survival in days of animals ultimately dying.

Normally, this infection is transmitted by the bites of mosquitoes. If virus is introduced subcutaneously or intramuscularly into the experimental animal, it multiplies greatly outside the nervous tissue and appears in the blood in high titre without producing definite signs of infection. One of two things may then happen—uneventful recovery from this inapparent infection, or invasion of the nervous system and death. In the mouse, by using sufficiently young animals the latter event may be brought about in nearly 100 per cent of cases. If now even a single oral dose of mepacrine be given before virus, or soon after virus during the stage before invasion of the central nervous system has taken place, a very considerable number of animals is protected even when large infecting doses of virus are used (Tables V and VI). Mepacrine has no action whatsoever on the virus *in vitro*. It also has no effect against equine encephalomyelitis in the chick embryo, so that its action would never have been discovered but for the test in the mouse.

The action of mepacrine is a true antiviral one because titration of virus in the blood and organs of treated mice shows that growth of virus is wholely or partly suppressed, according to the dose administered (Fig. 1). After dosing with mepacrine, there appear in the cells of many organs deposits of tiny basophilic granules which stain blue with Giemsa's

solution. When unstained they are yellow, and they fluoresce in ultra-violet light; in other words, they have the general properties of an acridine and are presumably a metabolite of mepacrine[59]. They are particularly heavy in the cells of the reticulo-endothelial system, where of course trypan red also accumulates, and we have found that a number of high-molecular-weight polymers of very varied chemical nature, which after intravenous inoculation accumulate in the reticulo-endothelial system, also have some protective action in equine encephalomyelitis, though nothing like that of mepacrine. In other words the antiviral action of mepacrine appears somehow to be connected with the reticulo-endothelial system.

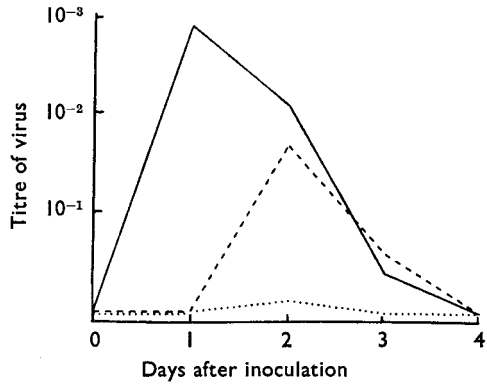
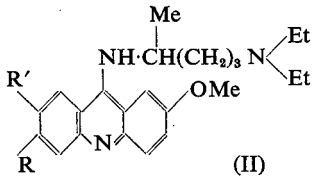


FIG. 1. The titration of equine encephalomyelitis virus in the blood of infected mice treated with two differing doses of mepacrine. The growth of virus is suppressed according to the dose given:

—— No treatment; - - - mepacrine 4 mg.; .... mepacrine 10 mg.

A number of acridines other than mepacrine have considerable antiviral activity[16], and all the most active produce basophilic granules. One of these is a nitro-acridine, and as has been seen some nitro-acridines are active against the large viruses. However, this particular one was not active against psittacosis or lymphogranuloma; in fact, we have not yet observed activity against both classes of virus in one and the same compound. How completely unpredictable these activities are may be seen from the results of varying the substituents R and R′ on formula II which is an acridine having the same basic side-chain and methoxy group. Compound II (R = Cl; R′ = H is mepacrine, which is active against equine encephalomyelitis in the mouse, but is not active against psittacosis or lymphogranuloma. Substitution of chlorine by a nitro group (II, R = NO_2; R′ = H) removes all activity for equine encephalomyelitis, but this compound is active against psittacosis and lymphogranuloma. When the nitro group is moved from the 6 to the 7 position in the acridine ring (II, R = H; R′ = NO_2) activity is regained against equine encephalomyelitis and lost against psittacosis and lymphogranuloma. None of these three compounds has any activity against viruses *in vitro*. When the nitro group at position 7 is replaced by an amino group (II, R = H; R′ = NH_2) all therapeutic activity against both large and small viruses is removed. The new compound, however, is remarkably active against all viruses *in vitro* and inactivates them in a very short time.



(II)

Metabolites of Mepacrine

Mepacrine also shows an effect against equine encephalomyelitis in the adolescent rat, but not in the guinea pig, the rabbit, the chicken or the monkey. When a drug is found to be active in one animal species but not in another, it often indicates that the metabolism of the drug differs in the several animal species, and that the therapeutic action is due wholely or principally not to the drug administered but to a metabolite of it. My colleagues and I have spent, and are spending, a great deal of time in trying to find an active metabolite of mepacrine—which might then be expected to be active in other animal species—so far without success. Of the total acridine present in the livers of mice dosed with mepacrine, about 94 per cent is unchanged drug[60]. The basophilic granules have been shown by suitable experiments not to possess appreciable therapeutic activity. However, several biological observations suggest that metabolism somehow comes into the picture.

TABLE VII

EFFECT OF SEX ON THE ACTION OF MEPACRINE IN EQUINE ENCEPHALOMYELITIS

Dose of mepacrine	Deaths in 30 mice	
	Male	Female
None	28 (5·2)	30 (5·8)
2 mg./20 g.	27 (5·9)	16 (6·8)
4 mg./20 g.	9 (7·2)	9 (8·1)

In the first place, if, after receiving a sub-optimal dose of mepacrine, the animals are dosed with SKF–525 A, the therapeutic effect is greatly diminished. SKF–525 A is a substance which interferes with the activity of certain enzyme systems in the liver which are responsible for metabolising and detoxicating drugs; its reported effect is to prolong greatly the action of drugs such as barbiturates by preventing their metabolism to inactive compounds. Of course, if it were a metabolite which was the active substance, SKF–525 A would then be expected to diminish the effect of the drug by preventing formation of the metabolite, which is precisely what it appears to do with mepacrine.

Secondly, by feeding the animals with tryptophane, which greatly increases the amount of certain enzymes in the liver, the effect of mepacrine is enhanced.

Thirdly, the effect of mepacrine is considerably affected by sex, which in itself is responsible for considerable differences in metabolism. Thus, with diminishing dosage of mepacrine, the protective effect wears off in the male at a dose which is about twice that which is minimally effective in the female (Table VII). Some of the sex and other hormones also materially affect the protective action of mepacrine. These observations are not yet complete, but they reinforce the suggestion made earlier, that in the chemotherapy of infective agents so intimately parasitic as viruses we may find that constitutional and nutritional factors play a not unimportant part.

In the United States a great deal of work has been carried out on the influence of nutritional factors in virus diseases, and particularly on the effects of depriving the animals of essential vitamins, mineral salts, amino acids, or just food in general. The results have been reviewed elsewhere[61],

and cannot be considered in detail here. Often the nutritional deficiency has apparently increased resistance to one or other virus, in the sense that the deprived animals have survived, on an average, longer than have those normally fed. In some at least of these experiments, the condition of the animals at the time of infection has been far from satisfactory, and we recall that an animal in an unsatisfactory state of health from one of several causes may be an unsuitable host for the full multiplication of a virus. That some of the experiments have no other interpretation is shown by the fact that removal of the deficiency has promptly led to development of the virus disease. There is one report, however, which if confirmed would seem to be of considerable interest. O'Dell and his colleagues[62] observed that by administering ribose or desoxyribose nucleic acids, especially in conjunction with a diet high in protein, such as one containing 60 per cent casein, they could confer a high degree of protection on mice infected with the neurotropic MM virus. They suggested that in the parasitised nerve cells of the normally fed animals the virus monopolised nucleic acid and protein and damaged the cell by leaving none available for normal cellular functions. By administering excess protein and nucleic acid they felt that they had provided a sufficient surplus to satisfy the requirements of both cell and virus and so to minimise the damage ordinarily ensuing from the presence of the latter.

Thus we see that a few diseases caused by the smaller viruses may be influenced to the advantage of the experimental animal, though sometimes by measures far removed from those of conventional chemotherapy. It seems not impossible that further research into the precise mechanism of these effects may provide information leading to effective chemotherapy in the future.

References

1. Francis, Martin, Rose and Spinks, *Brit. J. Pharmacol.*, 1954, **9**, 437.
2. Cornforth, Hart, Nicholls, Rees and Stock, *ibid.*, 1955, **10**, 73.
3. Davies, Driver, Hoggarth, Martin, Paige, Rose and Wilson, *ibid.*, 1956, **11**, 351.
4. Stone and Burnet, *Aust. J. exp. Biol. Med.*, 1945, **23**, 205.
5. Drake, Hampil, Pennell, Spizizen, Henle and Stokes, *Proc. Soc. exp. Biol., N.Y.*, 1952, **80**, 310.
6. Greenlee, Terrill and Sloan, *Texas St. J. Med.*, 1951, **47**, 831.
7. Report, *World Hlth. Org. tech. Rep. Ser.*, 1953, No. 62.
8. LoGrippo and Hartman, *J. Immunol.*, 1955, **75**, 123.
9. Rose and Gellhorn, *Proc. Soc. exp. Biol., N.Y.*, 1947, **65**, 83.
10. Ten Broeck and Herriot, *ibid.*, 1946, **62**, 271.
11. Bedson, *J. clin. Path.*, 1956, **9**, 83.
12. Hurst, Peters and Melvin, *Brit. J. Pharmacol.*, 1950, **5**, 611.
13. Inkley, *ibid.*, 1953, **8**, 448.
14. Katz, *J. infect. Dis.*, 1956, **98**, 177.
15. Eaton, Allen and Wiener, *Proc. Soc. exp. Biol., N.Y.*, 1947, **66**, 141.
16. Greenhalgh, Hull and Hurst, *Brit. J. Pharmacol.*, 1956, **11**, 220.
17. Hurst, *ibid.*, 1948, **3**, 181.
18. Hurst, Landquist, Melvin, Peters, Senior, Silk and Stacey, *ibid.*, 1953, **8**, 297.
19. Moulder, Colon, Ruda and Zebovitz, *J. infect. Dis.*, 1956, **98**, 229.
20. Weiss, *ibid.*, 1950, **87**, 249.
21. Meyer and Eddie, *ibid.*, 1951, **88**, 109.
22. Schmidt and Sprockhoff, *Z. Hyg. InfektKr.*, 1953, **138**, 122.
23. Meyer and Eddie, *Antibiot. Chemother.*, 1955, **5**, 289.
24. Moulder, McCormack, Gogolak, Zebovitz and Itatani, *J. infect. Dis.*, 1955, **96**, 57.

25. Siniscal, *J. Amer. med. Ass.*, 1952, **148**, 637.
26. Bauer, *Nature, Lond.*, 1949, **164**, 767.
27. Hoyle, *J. Hyg., Camb.*, 1952, **50**, 229.
28. Hoyle, Jolles and Mitchell, *ibid.*, 1954, **52**, 119.
29. Frisch-Niggemeyer and Hoyle, *ibid.*, 1956, **54**, 201.
30. Hoyle and Frisch-Niggemeyer, *ibid.*, 1955, **53**, 474.
31. Wyckoff, *J. Immunol.*, 1953, **70**, 187.
32. Liu, Ch'ien, *J. exp. Med.*, 1955, **101**, 677.
33. Watson and Coons, *J. exp. Med.*, 1954, **99**, 419.
34. Henle and Henle, *ibid.*, 1949, **90**, 23.
35. Hoyle, *Brit. J. exp. Path*, 1948, **29**, 390.
36. Commoner, Lippincott, Shearer, Richman and Wu, *Nature, Lond.*, 1956, **178**, 767.
37. Hart and Smith, *ibid.*, 1956, **178**, 739.
38. Hershey and Chase, *J. gen. Physiol.*, 1952, **36**, 39.
39. Markham and Smith, in *The Proteins*, **2**, A, 1. Ed. by H. Neurath and K. Bailey. Acad. Press Inc., New York, 1954.
40. Matthews and Smith, *Advances in Virus Research*, **3**, 49. Acad. Press Inc., New York, 1955.
41. Ackermann and Maassab, *J. exp. Med.*, 1954, **100**, 329.
42. Tamm, *J. Bact.*, 1956, **72**, 42.
43. Tamm, Folkers, Shunk and Horsfall, *J. exp. Med.*, 1954, **99**, 227.
44. Tamm and Tyrrell, *ibid.*, 1954, **100**, 541.
45. Horsfall and McCarty, *ibid.*, 1947, **85**, 623.
46. Ginsberg and Horsfall, *ibid.*, 1949, **89**, 37.
47. Ginsberg and Horsfall, *ibid.*, 1951, **93**, 161.
48. Hamre, Brownlee and Donovick, *J. Immunol.*, 1951, **67**, 305.
49. Thompson, Minton, Officer and Hitchings, *ibid.*, 1953, **70**, 229.
50. Thompson, Price, Minton, Falco and Hitchings, *ibid.*, 1951, **67**, 483.
51. Bauer, *Brit. J. exp. Path.*, 1955, **36**, 105.
52. Shope, *J. exp. Med.*, 1953, **97**, 601, 627, 639.
53. Powell, Culbertson, McGuire, Hoehn and Baker, *Antibiot. Chemother.*, 1952, **2**, 432.
54. Cochran, Brown and Francis, *Proc. soc. exp. Biol., N.Y.*, 1954, **85**, 104.
55. Cochran and Francis, *ibid.*, 1956, **92**, 230.
56. Wood and Rusoff, *J. exp. Med.*, 1945, **82**, 297.
57. Hurst, Melvin and Peters, *Brit. J. Pharmacol.*, 1952, **7**, 455.
58. Hurst, Peters and Melvin, *ibid.*, 1952, **7**, 473.
59. Hurst, Snow and Roberts, *Brit. J. Exp. Path.*, 1955, **36**, 215.
60. Goodall, *Brit. J. Pharmacol.*, 1956, **11**, 215.
61. Hurst and Hull, *Pharmacol. Rev.*, 1956, **8**, 199.
62. O'Dell, Wright and Bieter, *J. Pharmacol.*, 1953, **107**, 232.