

Second edition

Principles of
Animal Virology

F. M. BURNET

Principles of ANIMAL VIROLOGY

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Second Edition



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Preface to Second Edition

Virology advanced with unprecedented rapidity between 1950 and 1958 and it may be that the next decade will show even greater progress. It is, however, at least as likely that we had passed through the second and last golden age of virology by 1959 — that the principles of experimental and epidemiological approach are now laid down and that medical virology now faces a new set of problems and responsibilities.

The etiologies are defined, the principles of immunization are well known, the routines of diagnosis, of vaccine production, of public health action, can run smoothly with no more research than is necessary to maintain intellectual health among the workers concerned.

The problems of the future that will concern the research man will tend to fall into two categories. The first problem will be to sort out the dynamics of infection by each of the main groups of viruses and in the process to keep pace with the evolution of viruses and virus disease. We can guess that there will be another new influenza A virus within 20 years and that another enterovirus will become a major cause of poliomyelitis within 50 years. The second problem will be to use suitable virus strains as tools for the analysis of cellular function. Here we must face the possibility that plant and bacterial virus strains will always provide much more readily manipulated model systems than any animal virus.

If this reading of the situation in virology is correct, or at least a justifiable one, then there may be more justification for a new edition of this work than a 4- or 5-year gap would ordinarily justify. My impression is that the past 5 years have clarified the principles of animal virology and that they can be expressed in more balanced and acceptable form than was possible only a few years ago. The tissue culture revolution has run its course and a new orthodoxy is established.

Nearly half the text of the first edition has been replaced and an effort has been made to bring more into proportion those topics of the first edition which were my own special research interest while it was being written. One chapter has been eliminated.

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Melbourne, Australia
November, 1959

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CHAPTER I

An Historical Introduction to Animal Virology

INTRODUCTION

At the present time the study of viruses lies very near the heart of biology. They are the simplest organisms yet they exemplify clearly every major characteristic of living matter, replication with all that this entails, inheritable variation, selective survival within the appropriate ecological system, and no doubt an evolutionary history. At the same time we cannot forget that they have been responsible for two of the great historical pestilences, smallpox and yellow fever, for the great influenza pandemic of 1918-19, and for some of the most destructive diseases of domestic animals. If we accept the objectives of biological science as, on the one hand, the effective control of living organisms for the satisfaction of human needs and, on the other, a progressive understanding of the processes of life and their expression in logical and quantitative form, no branch of biology can claim to be of more significance.

As almost everywhere in the history of science, discovery commences with attempts to deal with immediate and pressing human problems. Smallpox in the eighteenth century was the most clearly defined of the causes of death amongst children and, for several reasons, the story of the early attempts to prevent smallpox is of significance for the eventual development of virology as a discipline of its own. In one sense smallpox is a disease that is particularly easy to understand. The visible lesions are highly characteristic and dramatically repulsive. It was easy to accept that the agent of the disease was in the "matter" of the pustules and, when "variolation" became the fashion, to prove this by inoculation of material to the skin of a susceptible child. Smallpox also left its mark; the pock-marked face was indubitable evidence of past infection and no one could escape recognizing the demonstration in every outbreak that second attacks of smallpox were virtually unknown. The transmission of an infectious disease by inoculation of an extract of diseased tissue and the development after infection of specific and long lasting immunity — these facts provided a basis from which all subsequent work on what we now know as virus disease was developed.

In the days before the development of bacteriological techniques all infectious agents were "viruses" and if they were to be studied at all at the experimental level the only available approaches were by such

crude inoculations and by microscopic search for possible organisms in blood and discharges.

The development of pure culture techniques by Koch and their application by his collaborators and successors was a tremendous advance. The classic approach to the investigation of infectious disease became the "isolation" of the responsible bacterium in pure culture and the development of methods by which its identity could be established. Once this was accomplished it had to be shown, first that bacteria of this type could be regularly isolated from persons or animals with the disease in question but not from healthy individuals or those suffering from other disease, second that appropriate inoculation of the pure culture in a susceptible animal would reproduce the disease condition. Although there were in fact many instances in which, for one cause or another, Koch's postulates could not be fulfilled, there was a time in the last quarter of the 19th century when it seemed that appropriate methods would eventually allow pure cultures of bacteria or fungi to be isolated and incriminated as the agents of all the infectious diseases. It soon became evident, however, that in addition to bacteria there were diseases due to protozoa and fungi, as well as a very considerable list which resisted any microscopic or cultural demonstration of a responsible microorganism. Beijerinck's (1899) early demonstration of the passage of the "virus" of tobacco mosaic disease through a porcelain filter, following Loeffler and Frosch's (1898) similar work with foot and mouth disease of cattle, brought the concept of filterability to the fore and the name "filterable viruses" became current.

The modern development of animal virology has been very largely in the years since 1925 and it is convenient to discuss that development from a background of the knowledge available in the period 1920-25. At that time well defined methods for the diagnosis of bacterial, protozoal, and mycotic diseases had been developed. Microscopic and cultural methods for the detection of pathogens were well advanced and in many instances serological tests were also available. Interest in bacteria as microorganisms with problems of nutrition and variation quite apart from their function as agents of disease was becoming more widespread.

At that time the "filterable viruses" were essentially the still uncharacterized agents of those diseases, clearly of infectious nature and, in some instances, transmissible by the inoculation of tissues or fluids from the diseased subject to experimental animals, for which no morphologically or culturally defined microorganism could be held responsible. Scientific interest in viruses necessarily remained wholly at the medical or veterinary level. There was, however, d'Herelle's discovery of bac-

terioophage in 1917 and following the publication of his first book on the subject in 1921 many bacteriologists were attracted to the study of this phenomenon. In time, these studies had a great deal of influence on the development of ideas in regard to animal viruses, but in the twenties there were few who accepted d'Herelle's view that "the bacteriophage" was a virus and, on the whole, the subject remained an isolated bacteriological curiosity without influence on the general development of biology until about 1930.

The history of research on virus and virus disease from this period becomes essentially a history of the development of new technical methods and of the emergence of new concepts as the implications of the technical discoveries were realized. For obvious reasons, technical developments proceeded differently in the three great domains within which virology has developed. Botanists and plant pathologists have developed their own approach to the plant viruses; the animal viruses responsible for disease in man, other mammals, and birds were at first the province of the medical or veterinary bacteriologist, while from the nature of the phenomena bacterial viruses were discovered and have always been studied in the bacteriological laboratory. In recent years and particularly with the increasing interest of chemists in virus problems, the animal, plant, and bacterial viruses tend to be considered together and it is a sign of the times that the Virus Laboratory opened at the University of California in 1952 should have been specifically built to handle all three types of virus. There are, however, some who doubt whether there is more than a superficial resemblance among animal, bacterial, and plant viruses. The essence of virus disease is the interaction between the virus and the host cell. It is an axiom of modern biochemistry that the fundamental synthetic and metabolic processes are common to all forms of living cells and it may eventually emerge that the process of virus multiplication is basically the same in all types of host cells. On the other hand, alongside the basic similarity there are such extreme differences in morphology, metabolism, and relation to other cells that wholly different technical approaches to the study of the three major fields of virus disease are required. In the present state of knowledge, work on plant viruses has little direct bearing on animal virology but the development of chemical work on tobacco mosaic virus has had a very big influence on the chemical approach to the smaller animal viruses.

If we adopt the current usage that by animal viruses we are essentially concerned with those pathogenic for warm-blooded vertebrates, man, other mammals, and birds, the important group of insect viruses has to be regarded as equivalent to a fourth main group almost as dis-

inct from the viruses pathogenic for vertebrates as are the bacteriophages. Insects, however, may eventually have an extremely important theoretical role to fill. They are important carriers of both plant and animal viruses and for some examples of both types there is evidence that multiplication occurs in the cells of the carrier insect. There is no hint, however, that either type of virus infection produces any symptoms in the carrier or any microscopically visible change. The classic virus diseases of insects are fatal infections producing gross changes of a type quite unlike the lesions of any vertebrate disease.

The bacterial viruses are now the best studied of all viruses, particularly the large particle coli-dysentery phages of which C16 and T2 are types. These viruses have a morphology and chemical composition that have no resemblance to those of any animal virus, yet functionally there is a very considerable resemblance to the viruses of the influenza group. There can be no question that work on bacteriophages has had and is having an important influence on the development of animal virology and any historical account of the science must pay due regard to work on bacterial viruses. In this book, however, we are primarily concerned with an attempt to understand the processes at work in virus diseases of man and other vertebrates. Only indirect reference will be made to the other groups of viruses but, in regard to the problems of virus-cell interaction, considerable use must be made of data from the bacterial viruses and any discussion of ecological factors in human and animal disease must include reference to virus multiplication in insects. The same rule will necessarily be followed in mapping out the landmarks in the historical development of animal virology.

ANIMAL VIRUSES: THE ETIOLOGICAL CONCEPT

In recent years tissue culture methods have greatly broadened the range of problems in virology that are available for study but in the past the commonest starting point was the occurrence of an outbreak of disease in man or some economically important animal. Even today this is the most urgent call to action and each episode will immediately raise an important series of problems. Perhaps the primary question is: Does the outbreak conform to the pattern of some already described and more or less clearly understood infectious disease? In other words, a diagnosis is required and since the development of the Pasteur-Koch doctrine of the specificity of infectious disease an etiological diagnosis has been demanded. Actually, even the bacteriologists have not found it easy to define the requirements for the linking of a given type of infection to a specific microorganism. An etiological diagnosis demands essentially that practical technical procedures are available by which the existence

of a microorganism in individuals suffering from the disease can be demonstrated in such a way that (a) the microorganism can be clearly differentiated from other types of microorganisms, and (b) it can be shown to be identical with or closely related to the organism known to be responsible for the same disease in other places and at other times. In the case of bacterial diseases, the requirements were fulfilled by the isolation and growth of the responsible bacterium in pure culture and the subsequent definition of the bacterial culture by application of all the classic bacteriological criteria from colony morphology to fermentation reactions, pathogenicity tests, and so on. It was inevitable that the progress of virus research should be along the lines mapped out by attempts to apply the sort of criteria that were laid down in "Koch's postulates" to virus diseases.

An accurate etiological diagnosis is by no means the only, or even the most important, requirement for the administrative and medical control of an outbreak of disease. Sometimes, as in the case of urban yellow fever, practical success in public health measures may long precede recognition of the true nature of the pathogen. Effective understanding and control, however, will normally demand a practical means of etiological diagnosis. There are several relatively trivial diseases — measles and chickenpox for example — in which the symptoms are usually so characteristic and the subsequent immunity so long-lasting and specific that simple clinical and epidemiological observation can provide, in our sense of the term, a fully adequate etiological diagnosis. At the other extreme, poliomyelitis and simple herpes of the lips for many years were not regarded as infectious diseases at all. Only sophisticated epidemiological study plus laboratory methods for recognizing the viruses allowed their elucidation.

At the practical level the one significant character of a virus is its power to produce disease; everything else is secondary. The first step in handling a virus experimentally has normally, therefore, been to attempt to produce a recognizable disease or lesion in some experimental animal. Pasteur's work on rabies was the first "set-piece" of virus research, and the development of Pasteur *et al.* (1881) of the method of inoculating rabies virus intracerebrally in rabbits marked the first systematic use of a small laboratory animal for the recognition and study of a virus. It was also significant in recognizing the usefulness of implanting the virus in a sheltered, unduly susceptible tissue.

Further advance was very slow in coming. The diseases now known to be of virus origin were studied by the available methods of bacteriology and histology without providing much that is now remembered. Technical methods of producing and handling vaccine lymph were

gradually improved and the first important step in the control of the second great pestilential disease, yellow fever, was taken at the beginning of the 20th century. The experiments of the U.S. Army Yellow Fever Commission (Reed, 1902) were conducted with human volunteers and established three vital points: (a) the virus was present in the blood of the patient for the first three days of the fever, (b) it was transmitted by *Aedes aegypti*, and (c) about twelve days must elapse before the mosquito can transfer infection. That knowledge provided all that was necessary for the control of urban yellow fever, but a real understanding of yellow fever had to wait until the virus was transmitted to monkeys and mice nearly thirty years later.

The Development of a Quantitative Approach

Quantitative methods of dealing experimentally with viruses were relatively late in developing. In the 1920's vaccinal lymph was tested in the rabbit skin either by scarification or intradermal inoculation at a 1:1000 dilution, a positive reaction being required before its issue for human vaccination. Harvey and Acton (1923) used serial dilutions of rabies virus for intracerebral inoculation in rabbits in tests of the relative content of virus in gray and white matter of infected brains. Probably the first good quantitative work on a virus disease was with foot and mouth disease. Waldmann and Pape (1920) showed the symptoms could be produced in guinea pigs by inoculation of the foot pad and by 1927 Bedson *et al.* had systematized titrations by the injection of serial dilutions into intradermal "tunnels" running longitudinally in the bare skin of the hind pad.

From the point of view of the development of academic virology, the most important phase was the sudden recognition around 1930 that mice were susceptible by intracerebral inoculation to many types of virus infection and that relatively accurate titrations could be made by the inoculation of serial dilutions of virus into adequate numbers of mice. The first systematic use of mice for this purpose was due to Andervont (1929) who showed that some strains of herpes simplex virus were pathogenic by this route. Soon afterwards Theiler (1930) extended the method to yellow fever virus and so opened up the modern phase of work on that disease. In the same year Hoyt and Jungeblut (1930) showed that rabies virus produced regularly fatal infections in the mouse brain. Shortly afterwards Alston and Gibson (1931) found that louping ill virus was equally lethal by this route, and the list was soon extended to Western equine and some other types of mosquito-borne encephalitis. Other methods of mouse inoculation were also exploited, peritoneal injection gave characteristic findings with psittacosis virus and

a little later intranasal inoculation was introduced for the study of influenza virus. By 1932 it was clearly recognized that the normal approach to the study of a human disease of unknown etiology was to obtain presumably infective material from the patient as early as possible in his illness and inject this into a wide variety of laboratory animals by several routes, always including the intracerebral. Experience was also making it clear that it was often necessary to passage from one animal to another for many generations before completely reproducible lesions of the intensity needed were produced. The problem of the adaptation of a virus to a new host was at first a purely practical difficulty in the development of a suitable experimental animal. Later it became a topic of great intrinsic importance.

The next major step was the utilization of the chick embryo as a source of susceptible tissues for virus growth. Woodruff and Goodpasture (1931) first used fowlpox on the chorioallantois and subsequently showed with Buddingh (Goodpasture *et al.*, 1932) that vaccinia would also multiply freely in this situation. The use of the chick embryo as a means of titrating virus was introduced in the work by Burnet and Ferry (1934) with fowl plague. This virus is acutely lethal for the embryo and any bacteriologically sterile material containing fowl plague virus could be conveniently titrated by using death of the embryo within two days as an index of infection. The viruses of Newcastle disease, vesicular stomatitis, and several of the encephalitides can be titrated in similar fashion. A considerable extension of the use of the chorioallantoic method became possible with the development of "pock counting" methods by Burnet (1936). The method has been used extensively with vaccinia (Keogh, 1936), herpes simplex virus (Burnet and Lush, 1939; Shaffer and Enders, 1939), and to a lesser extent with myxoma (Lush, 1937), ectromelia (Burnet and Lush, 1936), and several adapted influenza virus A strains.

A very important development in 1938 was Cox's use of the yolk sac for the growth of the rickettsiae of Rocky Mountain Spotted Fever and typhus (Cox, 1938). This very rapidly became the standard method for the laboratory study of all rickettsiae and completely displaced the use of the chorioallantois. Earlier work by Zia (1934) with typhus and by Burnet and Freeman (1937) with Q fever had shown significant multiplication on the chorioallantois with the production of characteristic lesions. The yolk sac was also found to be the site of election for growth of the psittacosis-lymphogranuloma venereum group of viruses (Rake and Jones, 1942). All these agents are microscopically visible in stained smears and evidence of infection could be obtained either by

observing death of the embryo or by examining smears of the yolk sac after an appropriate time of incubation.

The next important series of applications of chick embryo techniques grew out of work in Goodpasture's laboratory by Buddingh and Polk (1939) on the localization of bacteria and herpes virus in the various organs of the embryo. Their use of amniotic inoculation stimulated Burnet (1940) to introduce influenza virus by this route. This led to the development of the standard method for the primary isolation of influenza virus and by the end of 1941 to the general use of the allantoic cavity for the cultivation of influenza viruses. The technical approach was immensely simplified by the discovery of hemagglutination (Hirst, 1941; McLelland and Hare, 1941). This allowed the fact of infection to be established by testing a drop or two of allantoic fluid for hemagglutinin. The chick embryo thus became the standard experimental animal for all types of influenza viruses and for the related hemagglutinating viruses of Newcastle disease, fowl plague, and mumps.

In 1948 Dalldorf and Sickles made the completely unexpected discovery that the feces from two children suffering from mild poliomyelitis in the little town of Cocksackie, New York, contained a virus pathogenic for suckling mice, but quite inactive on older animals. This led to the rapid uncovering of a horde of more or less similar viruses from human throat secretions and feces. At least 30 serological types are known and there are many functional differences within the group which has become known as the Cocksackie virus group. They are occasionally responsible for a variety of relatively trivial febrile affections — of which Bornholm disease, epidemic pleurodynia, is the best defined clinically — but in most instances are harmless commensals. Other viruses previously studied in other hosts may be highly pathogenic for suckling mice and the method has found important uses in work on foot and mouth disease.

Finally, we have to record the emergence around 1950 of tissue culture methods as an effective tool for virus isolation and study. The special applicability of tissue culture to work with polioviruses was recognized by Enders and his collaborators (Robbins and Enders, 1950) and rapidly became the standard method for all types of laboratory manipulation and vaccine production. Cells from various human tissues were first used but monkey testis and soon afterwards monkey kidney became the standard source of cells, not only for polioviruses but also for a wide range of other agents.

Tissue culture techniques between 1950 and 1957 worked a virtual revolution in virology and allowed the isolation of an almost embarrassing flood of "new" viruses from human material. In most laboratories, it has displaced almost completely the older methods for the titration

and characterization of viruses and most fundamental studies on virus-cell interaction are now carried out in tissue culture. There are certain disadvantages to be noted and weighed against the outstanding convenience and wide potentialities of the new methods. In the first place viruses which are difficult to handle in tissue culture tend to be neglected in favor of those which grow readily, and there may be too great a readiness to transfer findings from tissue culture studies of virus-cell interaction to the situation in the infected host. A more specific disadvantage arises from the rather frequent presence of contaminating viruses or pleuropneumonia-like organisms in monkey kidney and other cultures.

A few of the developments based on tissue culture that have been of importance for the general development of virology may be outlined here.

1. *The Use of Continuing Cell Lines*

Workers in the field of cancer research had known for some time that pure lines of cells, usually of malignant origin, could be carried indefinitely in tissue culture. It was obviously desirable to examine whether such pure lines could serve as a substitute for monkey kidney cells that must necessarily be obtained at each stage from the living animal. Two cell lines that have been extensively used are L (from mouse fibroblasts) and HeLa [from an epidermoid carcinoma of the cervix (Gey *et al.*, 1952)]. The L strain (Earle, 1943) underwent malignant change in the process of adaptation to continuing culture. Scherer (1953) showed that herpes and pseudorabies viruses would multiply on L cells but HeLa cells, being of human origin, have found more widespread use in medical virology. Scherer *et al.* (1953) found that poliovirus would grow readily on HeLa cells. Gross cytopathogenic changes were produced and in almost all respects the HeLa cells were as suitable as those from monkey kidney. Hopes that many different types of continuing cell lines could be obtained from various human tissues with corresponding suitability for the growth of different viruses have not been fulfilled. Reasons for this are still obscure. In fact, there has been a recent wave of scepticism about many of the claims to have initiated stable cell lines in view of the demonstrable ease with which contamination by cells from established lines can take place in the laboratory. Perhaps the most greatly needed technical advance at the present time is to devise a way by which cells can multiply indefinitely *in vitro* without loss of most of their initial individuality.

First steps have been taken in the logical development of tissue culture growth of viruses toward continuous bulk growth for vaccine production by the use of suspended cells (Earle *et al.*, 1954; Graham and Siminovitch, 1955; Girardi *et al.*, 1956). No large scale use has yet

been reported, probably because of the continuing disinclination to use any cell line which is known or suspected to be of cancerous nature for the production of material to be used for human inoculation.

2. *Demonstration of Virus Action on Tissue Culture*

Although it had been known since the work of Maitland and Maitland (1928) that vaccinia virus could multiply at the expense of fowl kidney cells suspended in a simple medium, any effective use of tissue culture in virus research demanded that the fact of virus growth should be demonstrable in the tissue culture itself. Using the virus of Western equine encephalitis, Cox (1936) showed that necrotic changes were produced in tissue cultures of chick embryo cells and by a quantitative elaboration of the method, Huang (1943) was the first to titrate a virus in tissue culture. He found that necrosis of chick fibroblasts was produced by a given sample of WEE virus to the same titer as was observed in normal titrations made in mice or chick embryos. Since then the commonest index of virus action in tissue culture has been the cytopathogenic effect (CPE) as observed by low power microscopy. What is essentially a method of demonstrating CPE on a quantitative and macroscopic scale has developed from the pioneering work of Dulbecco and Vogt (1954) on plaque production by animal viruses. A flat glass surface is covered with a continuous monolayer of actively metabolizing cells and inoculated with a few infective units of an appropriate virus. After an appropriate period to allow virus particles to be taken up by the cells, excess fluid is removed and convection and mechanical currents stopped by the addition of a layer of melted agar gel. On continued incubation each primarily infected cell serves as a center from which adjacent cells can be infected, leading eventually to a plaque a few millimeters in diameter formed by necrotic cells in the middle of the general "lawn" of healthy cells. The method provides a most versatile quantitative technique for a wide variety of virus studies.

Metabolic changes of various types are taking place in healthy tissue culture cells which are modified or cease when cell death results from virus action. There are therefore many possible ways of demonstrating the presence of virus by chemical means. In practice the only widely used method depends on pH changes (Enders *et al.*, 1949; Salk *et al.*, 1954), cultures in which virus growth has caused cell necrosis being less acid than healthy tubes.

As a by-product of work with influenza viruses, most of which have little cytopathogenic effect on standard types of tissue culture, another method of detecting growth has been introduced. "Hemadsorption viruses" are detected by the fact that a layer of infected cells when