VIROLOGICAL TECHNIQUE

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FOREWORD

The great advances in knowledge made since the beginning of the tissue culture era have raised ever-widening ripples of interest in different facets of virology. Perhaps the most remarkable have been those dealing with the structure and function of viral components.

It is, however, appropriate to recall that viruses first came into prominence from their association with human, animal and plant disease. Man's mastery of virus diseases is by no means yet complete. In many laboratories time and thought is given to the more prosaic but still pressing problems of the isolation and identification of pathogenic viruses and the measurement of immune responses to them. Many of the techniques used in these investigations are widely known but the published description may on occasion omit just those practical details of most value to the student.

This manual, by authors of considerable practical experience, has the aim of providing a straightforward and reliable guide to laboratory practices in everyday use. Its purpose is clear. It will not replace experience gained at the laboratory bench but should go a considerable way in assisting its progress.

A. D. MACRAE

PREFACE

During the last three decades virology has increased in its scope and work on viruses and virus diseases is now carried out at many centres. Because of this rapid development and because new methods are continually being introduced it has proved difficult to compile a text book on Virological Technique which is ' up-to-date '.

We hope the contents of this book will be of value to both the newcomer to virology and the experienced laboratory worker. We would be grateful to receive suggestions or criticisms, as we feel sure that much has been left out.

It has been impossible to acknowledge the source of many methods, as these have been developed and modified over the years and the origins are now unknown. Where specific works have been mentioned they are referred to in the text or bibliography.

We would like to thank those commercial organisations who kindly lent us blocks or illustrations. We would also like to thank Mrs. B. Knight and Mr. C. Maclean, F.I.M.L.T., for the drawings, and Mr. C. Sutton for a number of photographs. Our thanks are also due to Mr. A. C. Taylor, F.I.S.T., who corrected the typescript and made valuable suggestions. We are grateful to Mr. J. A. Rivers of our publishers for his help and extreme patience. We acknowledge with thanks the help given by Dr. W. H. Hambling on the entero- and respiratory viruses and our thanks also to Dr. J. S. Porterfield for allowing us to use much of his material on the Plaque Technique. We are grateful to the World Health Organisation for permission to use a number of photographs from their Bulletin. We would offer our thanks to Dr. A. D. Macrae who kindly agreed to write the foreword to this book.

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CHAPTER 1

INTRODUCTION TO VIROLOGY

Viruses have probably been with us since life itself began and are known to have been the cause of diseases for centuries. There is an account of an epidemic which took place in China in the tenth century B.C. which was almost certainly smallpox. Viruses have been the cause of the great historical pestilences, e.g. smallpox and yellow fever, and influenza virus was responsible for the pandemic of 1918. The first active worker in virology was Jenner who conducted his famous experiments in vaccination against smallpox using cowpox material. Immunisation had been carried out using pustular material from mild cases of smallpox for the previous 50 years in England, having been introduced from the East where it had been practised for centuries.

The first step in the discovery of viruses was in 1892 when work done by Iwanowsky indicated the presence of a filterable agent in connection with a disease of tobacco plants. Although Iwanowsky was not fully convinced by his results, Beijerinck (1898) repeated these experiments and carried out further experiments which proved the presence of an agent which was filter-passing and also capable of multiplication. About the same time Loeffler and Frosch accidentally discovered the existence of the virus causing foot-and-mouth disease of cattle, and in 1901 Reed and his colleagues proved the existence of yellow fever virus.

For many years after the discovery of tobacco mosaic, foot-and-mouth, and yellow fever viruses, very little more was discovered except that other types existed and that they caused certain diseases. The reason for this lack of information was the false impression that viruses were too small to be examined microscopically or to be susceptible to centrifugation. It was not until the late 1920s that vaccinia virus was concentrated by centrifugation. This gave sufficient virus material to be analysed but nothing was attempted with the smaller viruses as it was felt that only the large viruses, similar in size to vaccinia, could be sedimented. At about this time (1917), d'Herelle demonstrated the presence of bacteriophage and although he was convinced it was a virus, it remained a 'bacteriological curiosity' until 1930 when it was used as a prototype of virus cell activity with great success. Elford's experiments (1931) with the filtration of viruses through collodion membranes of graded pore sizes, showed that they ranged in size from 10–700 mμ. The sizes of the various viruses measured by Elford have, on the whole, been confirmed by more recent work using the electron

microscope. Elford's work initiated a fresh outlook on what could be done with viruses and has led us to the present state of virology.

From this short historical summary it is apparent that virology is one of the youngest of the biological sciences and has really developed over the last 30 years. Anyone interested in a more detailed study of the development of virology should read *Viruses and Man* by F. M. Burnet (1955) and *Beyond the Microscope* by K. M. Smith (1957).

Characteristics of Viruses and Rickettsiæ

At one time the definition of viruses was mainly concerned with the facts that they could not be seen under the microscope, and that they were capable of passing through bacterial filters. The first part of the definition was inaccurate and organisms other than viruses are capable of passing through bacterial filters (cf. L forms of bacteria). Any definition applied to viruses must be fairly general as follows: 'a submicroscopic organism which will multiply only within a living cell and does not multiply by binary fission'. Rickettsiæ, although usually in the province of virologists, are there because of the rickettsiæ's fastidious growth requirements, which are supplied only by living cells. The rickettsiæ multiply by binary fission, are sensitive to antibiotics and their structure is similar to that of bacteria. These facts group the rickettsiæ with bacteria rather than with viruses.

Viruses have been divided for convenience into three groups, which arise from the different branches of biology dealing with them:

1. Animal viruses.
2. Bacterial viruses (Bacteriophage).
3. Plant viruses.

The animal viruses are naturally of major interest to medical laboratories, both diagnostic and research, and the work done on them has been concentrated mainly on the diagnosis and prevention of virus diseases. This book will be mainly concerned with the animal viruses, the bacterial and plant viruses being mentioned briefly to give an idea of the valuable information that has been gained from them and its application to medical virology.

Growth of Viruses

The growth cycle of animal viruses has been studied using the knowledge gained from investigations into the multiplication of bacteriophage in bacteria. It may help the explanation of animal virus growth to discuss the various stages in the phage/bacterium system. Adsorption of the phage to the bacterial wall is followed by the injection (action is similar to that of a spring-loaded needle) of phage nucleic acid, leaving the protein coat of the bacteriophage attached to the exterior of the bacterial wall. After entry of the nucleic acid, the next stage is

the 'eclipse phase'; this is very important. It is at this stage that the infective phage disappears, i.e. on breaking open the bacterium no infective phage is available. During the 'eclipse phase' which lasts for about 10 minutes, production of the component parts of phage is in progress. Each type of component part forms its own pool and it is by taking a unit from each of these pools that a bacteriophage is formed. On completion of the bacteriophage (approximately 100 bacteriophage per cell), the cell wall is burst (lysed) by the bacteriophage, which are then able to infect another bacterium.

The adsorption of animal viruses appears to be similar to that of bacteriophage, the virus attaching to the cell wall at specific receptor areas on the cell surface. Cell penetration mechanism of the virus is not known; it possibly occurs with the aid of viral enzymes which are capable of digesting the cell wall; there is no need for the injection method of the bacteriophage as tissue cells are not as tough and firm as bacterial cells. Once inside the cell, virus is not susceptible to the action of antibodies, either naturally or artificially induced. The 'eclipse phase' in animal viruses is similar to, although not identical with that of a bacteriophage, i.e. on breaking open an infected cell, no infective virus is found; with animal viruses 90% of the virus goes into eclipse, the remaining 10% being recoverable. This recoverable virus appears to be normal but, if left in the cell, it does not seem to multiply. The next stage is the production of the component parts of the virus; this occurs in different areas of the cell. Using the influenza virus as an example, the S antigen component is formed predominantly in the cell nucleus about 3 hours after infection, and the hæmagglutination factor component is produced mainly in the cell cytoplasm and is noticeable 4 hours after infection. After all the component parts have been produced, they join together to form virus particles and make their way to the cell membrane, becoming fully infective virus just as they pass through the membrane. The release of infective virus is by extrusion and may occur over a period of hours without any obvious effect on the cell (cf. phage).

Purification of Virus

The original work on the purification and chemistry of viruses was carried out mainly with plant viruses, due to the relatively large amounts of virus available and to the comparative ease in separation of virus from plant material. Tobacco mosaic virus is an example of a plant virus which has been extensively investigated because of the amount of virus available, e.g. 10% of the dried weight of the infected plant; it can be purified without significant decrease in viral activity, giving a homogeneous product. Many of the plant viruses, including tobacco mosaic virus, can reach such a high level of purity that they can be crystallised, each virus crystal forming a characteristic shape. Some of

these crystals have been examined by electron microscopy and have proved to be made up of rods or of regular spherical particles, and presumably each rod or sphere is an infectious virus particle. Tobacco mosaic virus crystallises to microscopic needle crystals, which consist of rod-shaped viruses closely packed side by side.

Before any critical investigations can be carried out on viruses, it is essential that the virus is present in a pure and as concentrated a state as possible. Virus particles on release from infected cells, are naturally contaminated with cell material and there is a considerable amount of work involved in the removal of this contaminating material. It is possible that a decrease in viral activity could be caused by inactivation of the virus due to the purification method, it is therefore of prime importance to choose a method which will not inactivate the virus, e.g. incorrect temperature, pH, etc.

Most of the methods used in the purification of viruses have been borrowed from protein chemistry techniques. As viruses consist mainly of protein the salt precipitation methods of protein chemistry are used with some degree of success. After preliminary clarification with standard centrifugation, viruses can be precipitated by treatment with methyl, ethyl or butyl alcohol, or by treatment with specific concentrations of ammonium or magnesium sulphate. The viruses are then removed from the contaminating material still in suspension. There are also methods of purification involving specific characteristics of the viruses, e.g. (1) the adsorption of influenza virus to chick red cells, or (2) the adsorption of tobacco mosaic virus on to Celite (Johns Manville Co. Ltd.*).

1. A suspension of chick red blood cells is added to the influenza virus preparation and left at 4°C. The virus at this stage adsorbs on to the surface of the red blood cells. The red blood cells with virus attached are sedimented by gentle centrifugation, the supernatant is removed and the red blood cells are resuspended and incubated at 37°C. Incubation at 37°C releases (elutes) the virus from the red blood cells. These are then removed by centrifugation at low speed leaving a suspension of comparatively pure virus. This suspension can be concentrated by high-speed centrifugation.

2. A similar process is possible with tobacco mosaic virus, substituting Celite (a diatomaceous earth) for the chick red blood cells and altering the conditions of pH rather than those of temperature.

Other methods of virus purification available are (*a*) digestion of the contaminating material with enzymes which will not attack active virus, (*b*) a modification of the agglutination/adsorption technique of bacteriology, using antibodies prepared against a normal cell or tissue extract.

The methods just mentioned are chemical in nature and may there-

* Johns Manville Co. Ltd., 20 Albert Embankment, London, S.E.11.

fore alter the structure of the virus in some manner. It is for this reason that physical methods of purification are preferred. Physical methods are based mainly on the difference in size between the virus and the contaminating material. The methods most frequently used involve high-speed centrifugation. The viruses of tobacco mosaic, equine encephalomyelitis and influenza have been successfully purified using differential centrifugation. Purification by this method is especially suitable for unstable viruses as it can be carried out relatively quickly and at low temperature. Differential centrifugation is not successful where there is host material with a sedimentation rate near that of the virus.

Density gradient centrifugation is an excellent method of purification. The principle of density gradient centrifugation is simple. On a solution of sucrose or glycerol in a plastic centrifuge tube a small amount of virus suspension is layered. With enough time and sufficient centrifugal force each particle moves down the density gradient until it reaches its own density level. There the virus particles gather in a sharp band which can be collected. Other physical methods of virus purification include chromatography, differential filtration and dialysis. In the purification of viruses it is sometimes advantageous to use a combination of two or more of the above methods.

Physical and Chemical Structure

The size and shape of the various viruses has been derived from information gained by the following techniques:

1. *Electron microscopy*, with its related techniques, including chemical identification, e.g. specific enzymes, acting on the virus structure.
2. *Analytical ultra-centrifugation.*

Early work on the size of viruses was done using gradocol membranes.

The shape and size of animal, bacterial and plant viruses vary considerably. As a rule the animal viruses are spherical, bacterial viruses are sperm shaped with polyhedral heads, and the plant viruses are rod shaped. These are generalisations and there are brick-shaped and rod-shaped animal viruses. Some of the smaller viruses are so uniform that when sufficiently concentrated they form characteristic crystals, e.g. poliovirus forms tetragonal prisms.

THE SHAPE AND SIZE OF A FEW REPRESENTATIVE VIRUSES

Psittacosis group	Spherical	400 mμ
Tobacco mosaic virus	Rods	300 × 15 mμ
Vaccinia virus	Brick shaped	260 × 210 mμ
Poliomyelitis virus	Spherical	25 mμ

Observation on virus substructure has been carried out by mechanical and chemical disruption of virus particles. This has led to a clearer

understanding of virus structure and activity. Breaking up of bacteriophage by mechanical means gives a suspension of 'heads' and 'tails'. It was demonstrated that the 'head' cannot attach to bacteria on its own, and it can be further broken up into an outer protein membrane and its enclosed deoxyribonucleic acid. Fowl plague virus has been broken up using ether to give two components, the small particle being ribonucleic acid and the large particle consisting of the protein coat.

A considerable amount of work is being done on the geometrical arrangement of the sub-particles making up the virus, and of their significance in viral activity, the particles seen in the cell prior to formation of the virus probably being these sub-units. The sub-units have specific characteristics, e.g. the complement fixing particle of the Rift Valley Fever virus measures 12 mμ. A complete virus consists of a protein shell of sub-units surrounding a core of nucleic acid.

The chemical analysis of virus is carried out by using the micromethods of biochemistry to determine the nucleic acids, proteins, lipids, etc., which constitute an infective virus particle. Some of the methods used include spectrophotometry, electrophoresis, chromatography and the use of radioactive labelled isotopes of the elements necessary to make up the various constituents of a virus.

A few members of each of the plant, bacterial and animal virus groups have been closely investigated as to their chemical composition. The plant viruses are the least complex, being simple nucleoproteins consisting of protein and ribonucleic acid; the nucleic acid content ranges from 6–35% of the virus. Bacteriophage has been thoroughly examined due to its relative simplicity of purification, and it has been shown to contain protein and deoxyribonucleic acid. The animal viruses have proved to be exceptionally difficult to purify and this has possibly led to erroneous results. Animal viruses which have been studied extensively are vaccinia, influenza and poliomyelitis. These have been shown to consist of nucleic acid (ribose or deoxyribose), protein, carbohydrates other than those present in the nucleic acids, and lipids. The lipid present in the virus is of the same type as is found in the host cell, and in fact virus composition varies, depending on the type of host cell in which it is grown.

CHEMICAL COMPOSITION OF SOME ANIMAL VIRUSES (%)

	Protein	*Nucleic acid*	*Lipid*	*Carbohydrate*
Vaccinia	80–90	5 (D.N.A.)	5	5
Influenza	60–70	1 (R.N.A.)	25	5
Poliomyelitis	70–80	25 (R.N.A.)	—	—

Vaccinia and poliomyelitis, due to their low content of lipid, are insensitive to lipid solvents.

Tobacco mosaic virus has been broken down chemically into ribonucleic acid and protein. A considerable amount of work has been done on this virus and it has been shown using tobacco mosaic virus as the model, that the nucleic acid of a virus is the important factor in virus infection, and that the protein protects, transports and probably introduces the nucleic acid into the cell. Work on tobacco mosaic virus has also shown that nucleic acid which has been separated from the protein coat, is still capable of infection, thus showing that the nucleic acid carries the information necessary for the production of complete virus. An interesting and important experiment has been carried out in which the nucleic acid has been removed from a virus and joined with the protein of another virus. This 'new virus' is capable of infecting a cell; the virus progeny of this infection is of the virus from which the nucleic acid originated. For a more detailed text and references to the purification and structure of viruses, *Viral and Rickettsial Infections of Man* (Rivers and Horsfall, 1959) should be consulted.

Transmission of Virus Diseases

Viruses are discharged from infected patients a few days before the onset of illness, until a week or in a few cases months after the recovery from the illness. Viruses are found in any of the following sites, depending on the particular disease: skin, nasal/pharyngeal secretions, urine, fæces, blood, etc. Therefore infection can be transmitted by direct contact, inhalation, fomites, food or drink, biting insects such as mosquitoes, lice, fleas, etc. In some cases the insects merely act as 'flying fomites' but in many instances they act as virus reservoirs and as a part of the virus life cycle.

As with bacterial infections, three states of infectivity exist:

1. The patient showing obvious signs of infection is prevented from spreading disease due to being immobilised in bed, except for the few days prior to the symptoms.
2. Sub-clinical infections with the patient being free to spread illness while feeling 'under the weather', e.g. the common cold.
3. Healthy carriers—many fatal outbreaks of influenza and measles have occurred in isolated communities after the introduction of 'healthy' visitors.

Spread of Virus

Direct Contact. Amongst the virus diseases transmitted by direct contact are lymphogranuloma venereum, inclusion conjunctivitis and warts which can be transmitted from one individual to another or from one part of the body to another. In the case of children whose hands

may have been in contact with infected fæces, there may be spreading of polio and similar viruses.

Droplets. The droplets expressed on the coughing and sneezing of patients suffering from influenza, colds, exanthemata (measles, smallpox, etc.) are certainly responsible for cross-infection. It is not known how long virus remains viable under the conditions of expressed droplets.

Fomites. Clothes and similar articles which have been contaminated with excreta or secretions containing virus, are sources of infection. Apart from these there is a risk of being infected with warts and other skin diseases involved in the use of communal gymnastic equipment and from walking barefoot on the floors of gymnasia and swimming baths. Swimming baths are possible sources of poliomyelitis and inclusion conjunctivitis ('swimming bath conjunctivitis').

Animal Infection. Animal diseases which are directly transmissible to man include rabies (dog-bite); psittacosis (a respiratory disease contracted from parrots and parakeets by inhalation of droplets or dust from infected birds), ornithosis (a similar disease contracted from pigeons and sea birds). Birds are also responsible for the mechanical transmission of foot-and-mouth disease and are important reservoirs of various types of encephalitis. A particular hazard to laboratory workers is the possibility of infection with B virus. This virus is serologically related to herpes simplex and is transmitted to man by the bite of an apparently normal monkey. The disease in man is almost invariably fatal.

Biting Arthropods. Many virus diseases, e.g. yellow fever, encephalitis, etc., are transmitted by bites from arthropods such as mosquitoes, lice, ticks, mites, etc. These arthropods become infected by sucking blood from an infected subject, while the subject is carrying the virus in his blood-stream (viræmia). After the blood has been ingested, it is 5–10 days before the arthropod can infect another host. During this 5–10 days' incubation the virus multiplies and makes its way to the salivary glands. It is at this stage that the arthropod becomes infective. The arthropod shows no effects of the infection but it is a permanent carrier and in many cases the virus is passed in the eggs to succeeding generations. In some cases (myxomatosis) the arthropod, a mosquito, is not infected and merely transmits infection by mechanical means, e.g. contamination of its mouth by biting through lesions.

Induced Infections and Latency. A sub-clinical infection may be present in a host which, if undisturbed, clears up and comes to naught, but if a stimulus is applied the infection may flare up and become severe, e.g. tonsillectomy causing a flare-up of poliomyelitis.

Latent infections appear similar to the above phenomenon but, in fact, are chronic illnesses which flare up from time to time, e.g. herpes simplex infection occurs in childhood and then subsides; the virus is

then stimulated from time to time by various agents and causes characteristic sores. The latent virus survives in its non-infective state in the cell and is passed on from cell to cell on cell division, until it is activated.

BIBLIOGRAPHY

BEIJERINCK, M. W. (1898). Über ein Contagium virum fluidum als Ursache der Fleckenkrank heit der Tabaksblatter. *Zentr. Bakt. Parasit.*, II, **5,** 27–33.

BURNET, F. M. (1955). *Viruses and Man.* Pelican Books A265. Penguin Books Ltd., Harmondsworth, Middlesex.

ELFORD, W. J. (1933). Principles of ultrafiltration as applied in biological studies. *Proc. roy. Soc.*, B, **112,** 384–406.

D'HERELLE, F. (1917). Sur un microbe invisible antagoniste des bacillus dysentériques. *C. R. Acad. Sci.*, **65,** 373–375.

IWANOWSKI, D. (1892). Über die Mosaikkrank heit der Tabakspflanze. *Bull. de l'Acad. Imp. des Sci. de St. Petersbourg*, 3 n.s., 67–70.

LOEFFLER, F. and FROSCH, P. (1898). Berichte der Kommission zur Erforschung der Maul-und Klauenseuche bei dem Institut für Infektionskrankheiten in Berlin. *Zentr. Bakt. Parasit.*, I, Orig. **23,** 371–391.

REED, W., CARROL, J. and AGRAMONTE, A. (1901). Experimental yellow fever. Washington, D.C., U.S. 61st Congress, 3rd Session, Yellow Fever, 1911. Senate Doc. 822.

RIVERS, T. M. and HORSFALL, F. L. (1959). *Viral and Rickettsial Infections of Man.* 3rd Edition. J. B. Lippincott Co., Philadelphia.

SMITH, K. M. (1957). *Beyond the Microscope.* Pelican Books A119. Penguin Books Ltd., Harmondsworth, Middlesex.