TECHNIQUES IN EXPERIMENTAL VIROLOGY

Edited by

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PREFACE

The scientific community is showered from all sides today with "Advances in This", "Progress in That" or "Annual Review of The Other". To add to these yet another volume of collected papers might, therefore, be considered indefensible. The intention of the editor and his colleagues here, however, is quite different. We have each felt that there was a need for a source-book of techniques in virology. The diagnostic aspects have not been attempted so the term "experimental virology" more accurately describes the contents.

The techniques and methods used in the study of bacterial genetics and the phages would make up a volume in themselves, and indeed, there are current texts covering these topics. They have been excluded.

The subjects of plant, insect and animal viruses are covered in detail from the standpoint of the experimental methods used for the study of their growth *in vivo* or *in vitro*, their purification, analysis, assay, serology, and the determination of their interaction with the cell, and their ultra-structure, by electron microscopy.

Each contributor was given complete freedom to develop his topic at the optimum length, and to illustrate it as he thought fit. The only requirement was that the *how* should be expounded rather than the *why*. Some authors have been encouraged to give more detailed accounts than others, especially where the original data are widely spread in journals, and in time, in the scientific literature. Inevitably the subject has moved forward while the book was in preparation—and, equally inevitably, it took longer to prepare than was ever anticipated. For this delay the publishers are in no way responsible; the hold-ups occurred at chapter level, and the editor was too soft-hearted.

Inevitably, too, there is some overlap; where, for example, the methods used to prepare infective nucleic acids are similar for both plant and animal viruses they have been mentioned in both chapters.

It has been our intention to produce a book to be used in the laboratory rather than in the library; for those galloping enthusiastically into a new field rather than for those lying contentedly in the middle; for all virologists, plant, insect, or animal, for whom the selection of the right technique is as important as the choice of the right wife.

R. J. C. HARRIS

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CONTENTS

Chapter 4

INSECT VIRUSES

G. H. BERGOLD

Chapter 5

PURIFICATION OF ANIMAL VIRUSES

ROBERT CRAMER

Chapter 6

ANIMAL VIRUS TITRATION TECHNIQUES

ROBERT M. DOUGHERTY

Chapter 7

SEROLOGICAL TECHNIQUES

PAUL FISET

Chapter 8

HEMAGGLUTINATION

LEON ROSEN

Chapter 9

THE INFECTIVE NUCLEIC ACIDS OF ANIMAL VIRUSES

F. KINGSLEY SANDERS

Chapter 10

INTERFERENCE AND INTERFERON

J. S. PORTERFIELD

Chapter 11

TISSUE CULTURE TECHNIQUES

G. NEGRONI

Chapter 12

ULTRASTRUCTURAL STUDIES

A. P. WATERSON

Chapter 13

ELECTRON MICROSCOPY OF VIRUSES IN CELLS AND TISSUES

D. F. PARSONS

Chapter I

PREPARATION AND PROPERTIES OF PLANT VIRUS PROTEINS

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I. INTRODUCTION

The term "virus protein" was often used interchangeably with the word "virus" in the early literature on the chemistry of plant viruses. This usage can be traced back to the historic crystallization of tobacco mosaic virus (TMV) by Stanley (1935) and his conclusion that "Tobacco-mosaic virus is regarded as an autocatalytic protein which, for the present, may be assumed to require the presence of living cells for multiplication." Even after it was discovered that TMV and other plant viruses contain nucleic acid (Bawden *et al.*, 1936; Stanley, 1937), the term, "virus protein", continued for a few years to be used for "virus", partly because of precedent, and partly because the chemical findings

TABLE I

Size, Shape, and Approximate Composition of some Plant Viruses

Virus	Shape and size	Particle weight (avograms)	% RNA	% Protein	Reference
Alfalfa mosaic	rod-like, 20×55 mμ	5×10^{6}*	19	81	a, b
Broad bean mottle	spheroidal, 17 mμ	5.2×10^{6}	22	78	c, d
Bromegrass mosaic	spheroidal, 17 mμ‡	4.6×10^{6}	21	79	e
Cucumber 3 (and 4)	rod-like, 18×300 mμ	40×10^{6}	5	95	f; g
Potato X	filamentous, 13×520 mμ	35×10^{6}	6	94	h, i
Southern bean mosaic	spheroidal, 28 mμ	6.1×10^{6}	21	79	j, k
Tobacco mosaic	rod-like, 18×300 mμ	39×10^{6}	5	95	l, m, n
Tobacco rattle	rod-like, 25×185 mμ	73×10^{6}	5	95	o, p
Tobacco ringspot	spheroidal, 26 mμ	6×10^{6}*	35	65	q
Tomato bushy stunt	spheroidal, 30 mμ	8.9×10^{6}	17	83	r, s, t
Turnip yellow mosaic	spheroidal, 28 mμ	5×10^{6}	37	63	j, u
Wild cucumber mosaic	spheroidal, 28 mμ	7×10^{6}	35	65	v, w

* Estimated from sedimentation constant.
‡ Estimated by comparison with broad bean mottle virus.
a, Bancroft and Kaesberg, 1960; b, Frisch-Niggemeyer and Steere, 1961; c, Bawden *et al.*, 1951; d, Yamazaki *et al.*, 1961; e, Bockstahler and Kaesberg, 1961; f, Knight and Stanley, 1941; g, Knight and Woody, 1958; h, Brandes, 1961; i, Reichmann, 1959; j, Kaesberg, 1959; k, Lauffer *et al.*, 1952; l, Williams and Steere, 1951; m, Boedtker and Simmons, 1958; n, Knight and Woody, 1958; o, Paul and Bode, 1955; p, Harrison and Nixon, 1959; q, Steere, 1956; r, Williams, 1953; s, Cheng and Schachman, see Schachman and Williams, 1959; t, de Fremery and Knight, 1955; u, Markham, 1951; v, Sinclair *et al.*, 1957; w, Yamazaki and Kaesberg, 1961.

showed that plant viruses consist predominantly of protein. However, the term "virus protein" is now properly and almost universally applied to the protein component alone, which is the way it will be used here.

All plant viruses studied so far have been found to consist solely of protein and ribonucleic acid (RNA), and hence are ribonucleoproteins. As shown in Table I, the RNA contents of plant viruses range from about 5% to 37%, but when related to particle weights, it can be seen (Frisch-Niggemeyer, 1956; Knight, 1963) that the absolute amounts of RNA present are less variable, namely about $1\text{–}4 \times 10^{6}$ avograms.

The study of plant virus proteins is generally predicated upon the prior acquisition of highly purified virus preparations. With respect to purification, each virus poses a separate problem. The details of various methods for purifying plant viruses have been given elsewhere (Steere, 1959; Knight, 1963). Hence, only some of the guiding principles will be summarized here.

Basically, the purification of plant viruses is a problem in protein chemistry, since these viruses consist predominantly of protein. Therefore, protein fractionating techniques are commonly employed. In addition, viruses are macromolecular substances, which means that

they can be sedimented in an hour or two in high gravitational fields (40,000 to 150,000 g) whereas many host cell proteins cannot.

The major purification methods can be placed in three groups (Knight, 1963) reflecting the use of different principles: 1. precipitation, 2. adsorption, 3. differential centrifugation (alternate low-speed and high-speed centrifugation). These methods are frequently supplemented with density-gradient centrifugation, electrophoresis, various extractions, and treatments with specific sera and with enzymes. The latter treatment depends for its success on observations that viruses are generally much more resistant to proteases and nucleases than are typical host cell constituents. Recently, partition in liquid two-phase systems has been added to the list of virus purification procedures (Albertsson, 1960).

The degree of homogeneity (often called "purity") of a virus preparation cannot be evaluated by any single test. A rigorous evaluation requires the application of as many tests as possible, and at least some of these must be combined with infectivity measurements in such a way that a relation between physical particles and biological activity can be established. Among the methods often employed for determining homogeneity of virus preparations are the following: 1. analytical centrifugation, with which information can be obtained regarding the size, shape, and density of the particles present; 2. electrophoresis, by means of which electrochemical homogeneity can be evaluated; 3. electron microscopy, which permits a direct visual appraisal of the particles present; 4. immunochemical tests, which are particularly useful in detecting host cell contaminants of a proteinaceous nature; and, to a lesser extent, 5. the constant solubility test; and 6. crystallinity.

II. Preparation of Protein from Purified Virus

The plant viruses most commonly studied by physical and chemical methods are rather stable nucleoproteins. The RNA component in all cases seems to be deeply embedded in the protein to which it may be partly attached by means of salt linkages (e.g. between basic amino acid residues and acidic phosphoryl groups), hydrogen bonds, and other secondary linkages, although the precise bonding arrangements between protein and nucleic acid are not known for any virus. The protein components of plant viruses, and probably of viruses in general, are comprised of hundreds of apparently identical subunits and these seem to form secondary bonds among themselves as well as with the nucleic acid. Therefore, plant viruses are more or less readily disaggregated into their protein and RNA components by agents which disrupt secondary

bonds, such as urea, detergents, heat, alkali, acid, and phenol. So far, TMV and some of its strains have proved to be readily dissociable into RNA-free, essentially native protein (as judged by solubility characteristics and capacity to reconstitute into viral rods), but the spheroidal viruses have been much more difficult to work with, tending to yield by the same techniques denatured protein or protein still associated with nucleic acid. Some of the methods which have been used successfully in preparing viral proteins will be given here.

A. ALKALI METHOD

The alkaline degradation method has been applied most successfully to TMV and its strains, beginning with the systematic studies of Schramm (1947a, b) and his colleagues (Schramm *et al.*, 1955) and continuing with the investigations of others (for example, Fraenkel-Conrat and Williams, 1955; Harrington and Schachman, 1956). The objective of this and other disaggregative procedures is to break secondary bonds without disrupting primary valence bonds. The reaction in the case of TMV can be represented as follows:

$$\underset{\text{M.W. } 40 \times 10^6}{\text{TMV}} \xrightarrow{\text{pH } 10.5} \underset{\text{M.W. } 52{,}593}{\text{"A-Protein"}} + \text{degraded RNA}$$

It should be noted that the "A-Protein" (alkali protein), as it was called by Schramm *et al.* (1955), does not represent the ultimate protein subunit of TMV, but rather appears to be a semi-stable polymer (trimer). The protein subunit of TMV has a molecular weight of 17,531 and this unit has a pronounced tendency to aggregate so that it is observed in monomeric form only under highly disaggregating conditions (Wittmann, 1959; Ansevin and Lauffer, 1959; Anderer, 1959a, b).

1. *Alkaline Buffers*

The type of alkaline buffer employed seems not greatly to influence the end result in the preparation of protein from TMV. Thus, borate, carbonate, and glycine buffers have all been used successfully. In fact, if provision is made for addition of alkali at intervals, no buffer is needed, and the disaggregation is accomplished by addition of sodium hydroxide to maintain the proper pH.

The following procedure can be used for preparing protein from TMV or its strains:

An aqueous solution of TMV at about 10 mg per ml is placed in a cellophane bag and dialyzed for 2–5 days at about 4° against 2 l of

0.1 M carbonate-bicarbonate buffer (21.2 g Na_2CO_3 in 2 l H_2O adjusted to pH 10.5 by addition of solid $NaHCO_3$). Undegraded virus and denatured material are removed from the protein solution by centrifuging the contents of the dialysis bag at 60,000–100,000 g for 1 h and discarding the pellet. The protein is then separated at room temperature from alkaline degradation products of the viral RNA by adding one volume of saturated ammonium sulfate to the supernatant fluid from the high-speed centrifugation. The precipitated protein is sedimented by centrifuging at about 5,000 g for 10–15 min. The precipitate is dissolved in water and reprecipitated by adding a half-volume of saturated ammonium sulfate. The solution and precipitation steps are repeated once more and the final solution of protein is dialyzed at 4° against several changes of distilled water. A small amount of aggregated material may be present at this stage and is removed by adjusting the pH to 7–8 with dilute alkali and centrifuging at 60,000–100,000 g for an hour. The final, clear solution of protein is stored at about 4° adding a drop or two of chloroform as a preservative. The ultraviolet absorption ratio of the maximum to the minimum (280/250 mμ) should be about 2.4 for TMV protein and somewhat higher or lower for various strains of TMV.

The use of alkaline buffer at 30° for 30 min has been found by Kaper (1960) to release RNA from particles of turnip yellow mosaic virus leaving mainly the protein shells (hollow virus particles), some of which are further degraded by the alkali. The protein shells, consisting of about 150 subunits (Harris and Hindley, 1961) can be used for a variety of chemical, physical, and serological experiments. Kaper's procedure for preparing the protein shells is as follows:

To one part of purified turnip yellow mosaic virus at about 34 mg per ml in 0.01 M phosphate buffer at pH 7 is added 2.4 parts of a 0.2 M, pH 12, K_2HPO_4–NaOH mixture to give a final virus concentration of 10 mg/ml. The reaction mixture is held at 30° for 30 min and then centrifuged at low speed to remove an inorganic precipitate (probably Ca and Mg phosphates). The opalescent supernatant fluid is then centrifuged at about 100,000 g for 2 h and the resulting pellet is dissolved in 0.1 M phosphate buffer at pH 7. The product is subjected to two more cycles of centrifugation (alternate high-speed and low-speed), discarding the supernatant fluids of the high-speed centrifugation.

2. *Amino-alcohols*

While the end products seem much the same when TMV is degraded by a variety of alkaline reagents, the rate of degradation of TMV may vary considerably depending on the reagent used. Newmark and Myers (1957) found that amino-alchohols of the general formula

$RR'\underset{\underset{NH_2}{|}}{C}—CH_2OH$ are more efficient in degrading TMV than alkaline buffers, such as described above, and their use substantially shortens the time required to prepare TMV-protein by alkaline degradation. A procedure using an amino-alcohol is as follows:

An aqueous solution of TMV at about 10 mg per ml is dialyzed overnight against 0.02 M ethanolamine at pH 10.5. The contents of the dialysis bag are subjected to centrifugation at 60,000–100,000 g for about an hour and the supernatant fluid is treated with ammonium sulfate, etc., in accordance with the procedure described above for alkaline buffers.

B. ACID METHOD

The proteins of some strains of TMV are too easily denatured at alkaline pH values to permit their isolation by alkaline degradation procedures. However, Bawden and Pirie (1937) and Stanley and Loring (1938) observed that strains of TMV are degraded by acetic acid and this observation was made the basis for a convenient method for preparing viral protein by Fraenkel-Conrat (1957).

To an aqueous, ice-cold solution of virus at 10–30 mg per ml is added 2 volumes of glacial acetic acid chilled nearly to the freezing point (about 17°). The mixture is kept in chipped ice for 30–60 min with occasional stirring. During this time the degradation of the virus is marked by a loss of opalescence and the appearance of a fine, granular precipitate of RNA. The nucleic acid is removed by centrifuging for 10–15 min at low-speed (5000 g) in the cold in tubes of such material as polyethylene or glass which are not soluble in 67% acetic acid. The water-clear protein solution is then placed in a cellophane bag and dialyzed at 4° for 2–3 days against several changes of distilled water. During this time (in the case of the proteins of TMV and its strains) the protein comes into its isoelectric range and is transformed into a milky suspension. At this point, the contents of the dialysis bag are transferred to a centrifuge tube and the protein pelleted by centrifuging at about 5000 g for 10–15 min. The supernatant fluid is discarded and the pellet is suspended in a volume of water which will give an estimated protein concentration of 10–30 mg per ml. The protein is dissolved by adding dropwise 0.1 M NaOH until a pH of 8 is attained. This is done at 0–4° (or often at room temperature) with stirring and may require an hour or two, after which the solution is centrifuged at about 100,000 g for an hour in order to remove the small amount of undegraded virus or denatured protein which may be present. The water-clear supernatant

fluid, which contains the protein, is stored at 4° after adding a few drops of chloroform as a preservative. The yield is 80% or better with TMV, but may be appreciably less with some strains.

Quantitative yields of protein were also obtained when the acetic acid method was applied to turnip yellow mosaic virus (Harris and Hindley, 1961), but the product was an insoluble aggregate, apparently caused by the formation of S–S linkages between the subunits released from the virus by action of the acetic acid. This aggregation can be prevented by converting cysteine residues into carboxymethylcysteine residues before treating with acetic acid. The modified procedure, which may prove generally useful for viral proteins containing more sulfur than TMV, is illustrated by the turnip yellow mosaic method (J. I. Harris and J. Hindley, personal communication):

To 2.5 ml of a solution of turnip yellow mosaic virus at 30 mg per ml in 0.004 M Tris-HCl buffer at pH 8.2 is added 5 mg of iodoacetate in about 0.5 ml of solution (5 mg of iodoacetic acid are dissolved in 0.5 ml water and adjusted to pH 8.2 with NaOH). The mixture is placed in a pH-stat set to maintain the pH at 8.2 at 30°, and 2–2.5 g of solid urea are added (i.e. enough to make the solution 6–8 M with respect to urea). The urea dissociates the virus sufficiently to permit the iodoacetate to react with the –SH groups and the reaction is allowed to proceed at pH 8.2 and 30° for 1 h. The reaction mixture is cooled to 4° and treated with 2 volumes of cold, glacial acetic acid. The RNA precipitates and after standing at 4° for 1 h is removed by centrifuging the mixture at 5,000–10,000 g for 20 min. The clear supernatant fluid is then dialyzed at 4° against several changes of distilled water. The protein precipitates but may be redissolved by adding a few drops of N NH_4OH. The protein solution may be stored at 4° with a few drops of chloroform added as a preservative, or it may be dried from the frozen state (lyophilized).

C. WARM SALT METHOD

The protein subunits have been released from the small, stubby, rod-like particles of alfalfa mosaic virus by treatment with warm 1 M salt as follows (Kelley and Kaesberg, 1962):

To alfalfa mosaic virus at 20–30 mg per ml in 0.01 M phosphate buffer at pH 7 is added an equal volume of 2 M NaCl and the resultant mixture is held at 45° for 20 min. (During the heating the solution becomes turbid, presumably because the released protein is less soluble than whole virus in M NaCl.) The reaction mixture is cooled immediately and centrifuged at low speed, which sediments the protein while the RNA and/or its degradation products remain in the supernatant fluid. The protein pellet is suspended in M NaCl and centrifuged, discarding