CHEMISTRY OF VIRUSES

Second Edition

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Preface

In 1963, the first edition of *Chemistry of Viruses* was published as a contribution to the series on viruses sponsored by Protoplasmatologia. An aim of the first edition was to review some major principles and techniques of chemical virology in a concise manner and to accompany this review with a compilation of pertinent references. It was anticipated that this exercise would be helpful to the author in his teaching and research and, hopefully, would be useful to readers as well.

The literature of virology has grown enormously since then, and it is even more urgent to have a succinct survey. In addition, few authors have attempted to integrate the findings pertaining to the various major classes of viruses (that is, animal, bacterial, and plant viruses) but, rather, have chosen to assemble large monographs dealing in depth with facts and fancies pertaining to specific groups of viruses. Such works are valuable for pursuit of particular topics but fail to yield a brief, integrated view of virology. The present edition of *Chemistry of Viruses* aspires to such a review.

A serious attempt was made to deal concisely with every major topic of chemical virology and to present examples from different classes of viruses. Numerous references are given to original articles and review papers as well as to selected books.

It is hoped that this type of presentation—a compendium of chemical virology with pertinent, selected references—will prove to be a helpful introduction to viruses for neophytes and a convenient reference to veterans.

The author acknowledges with gratitude the contribution of illustrations by several colleagues, who are cited with the illustrations they provided, and the work of E. N. Story in preparing some of the illustrations. He is also indebted to Maureen Rittenberg for her efforts in typing the manuscript.

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I.

Some Events Leading to the Chemical Era of Virology

Near the end of the 19th century, Dutch scientist Martinus W. Beijerinck performed some experiments that were to have far-reaching consequences in science. Working with the sap from leaves of mosaic-diseased tobacco plants, Beijerinck (1898a, 1898b; see also van Iterson et al. 1940) showed that the infectious agent causing mosaic disease was so small that it passed through exceedingly fine bacteria-retaining filters and diffused at a measurable rate through blocks of agar gel. To this unprecedentedly small pathogen, Beijerinck applied the terms "contagium vivum fluidum" (contagious living fluid), or "virus."

As early as 1892, the Russian scientist, Ivanovski, reported filtration experiments with infectious juice from mosaic-diseased tobacco plants, but he was not convinced that his results were valid. In fact, a year after Beijerinck's report, Ivanovski (1899) published a paper on mosaic disease in which he concluded from his experiments that this condition was a bacterial infection. The following excerpt illustrates this point: "Zwar sind die Versuche noch wenig zahlreich und der Prozentsatz der erkrankten Pflanzen gering; doch glaube ich, dass die Bakterielle Natur des Kontagiums kaum zu bezweifeln ist."

In Germany, Loeffler and Frosch reported in 1898 that foot-and-mouth disease could be transmitted to calves by intravenous injection of infective lymph which had been freed of bacteria by passage through a filter candle made of diatomaceous earth (kieselguhr). Experiments involving dilution of the lymph and serial passage virtually eliminated the possibility that the disease could be attributed to a nonreproducing agent such as a toxin. Loeffler and Frosch therefore concluded that the causal agent was able to reproduce in cattle and was so small that it could pass through the pores of a filter that retained the smallest known bacterium. They also suggested that the hitherto elusive agents of such diseases as smallpox, cowpox, rinderpest, and measles might belong to this group of tiny organisms.

During the first 30 years of the 20th century, following the lead given by the work on tobacco mosaic and foot-and-mouth diseases, many infectious agents were tested for their filterability. As a consequence, such diverse diseases as yellow fever, Rous sarcoma of chickens, rabies, infectious lysis

of bacteria, cucumber mosaic, potato X disease, and many others were classified in the newly recognized group of ultratiny disease agents, the "filterable viruses." To characterize these newly recognized disease agents better, many studies were made of the effects of various chemical and physical agents on infectivity. The results of these pioneer investigations have been well summarized by Stanley (1938).

While early interpretations of the mechanism of inactivation of viruses by chemical and physical agents were necessarily faulty as judged by more recent knowledge, nevertheless, the results did provide a foundation on which ultimately successful attempts to isolate and purify viruses could be built. For example, it became clear that protein denaturants, oxidizing agents, formaldehyde, strong acids or bases, and high temperatures were inimical to viruses, whereas the milder protein precipitants, low temperatures, and neutral pH could usually be employed without destroying infectivity.

A prelude of what was shortly to come appeared in the experiments of Vinson (1927) and of Vinson and Petre (1929, 1931) on tobacco mosaic virus (TMV). A series of experiments on infectious sap from mosaic-diseased tomato or tobacco plants was summarized by Vinson and Petre (1929) in the following manner:

> We have found that when precipitation of the virus is carried out under favorable conditions, with the proper concentration of safranin, acetone, or ethyl alcohol, the precipitation is almost complete. In each case the precipitate contains practically all of the original activity of the juice, and the virus concentration in the supernatant liquid is no greater than that obtained by diluting a fresh juice sample one thousand-fold. This, together with the fact that the virus is apparently held in an inactive condition in the safranin precipitate and is released when the safranin is removed, makes it probable that the virus which we have investigated reacted as a chemical substance.

In a subsequent publication (Vinson and Petre 1931) the supposed nature of this chemical substance was postulated to be enzymic, largely on the basis of viewing the viral multiplication process as an autocatalytic phenomenon and on experimental hints that the virus might be proteinaceous. The chief clue that the virus might be associated with protein was an observed increase in nitrogen content as the infectious fraction was separated from the bulk of impurities associated with it, although the observations that the infectious principle moved in an electric field and was precipitated by protein precipitants were also consistent with the protein hypothesis.

Interest in TMV increased considerably when Vinson described infectious crystalline preparations of TMV at meetings of the American Association for Advancement of Science in 1928 and 1930, and published the relevant experiments in some detail in 1931 (Vinson and Petre 1931).

These crystalline preparations were obtained by treating infectious tobacco juice with acetone to get a precipitate, which was dissolved in a small amount of water. To this concentrated solution, acetic acid was added to pH 5; then acetone was added slowly with constant stirring until a slight permanent cloudiness appeared. When stored in the icebox, crystalline material often, but not always, separated out. Such crystalline material, when obtained, was described as "moderately active" (infectious), but as a protein preparation it was of dubious purity since about 33 percent was found to be ash (largely calcium oxide). Nevertheless, the finding was acclaimed, somewhat prematurely, in an editorial in the *Journal of the American Medical Association* (1932) in part as follows:

> Possibly the reported successful crystallization of the etiologic factor of mosaic disease of tobacco may be regarded by future medical historians as one of the most important advances in infectious theory since the work of Lister and Pasteur. The announcement of the isolation of a crystallizable pathogenic enzyme necessarily throws doubt on the conception that poliomyelitis, smallpox, and numerous other "ultramicroscopic infections" are of microbic causation. The apparent evidence that a specific protein, which in itself is incapable of self multiplication, may function as a disease germ when placed in "symbiosis" with normal cells seems to furnish experimental confirmation of several highly speculative theories relating to vitamins, hormones, and progressive tissue degenerations.

From the foregoing, it is evident that Vinson and associates contributed substantially to the chemical elucidation of TMV, but fell short of a definitive identification of the infectious agent. Hampered by persistent impurities in the preparations, uncertain biological assays, and variable but great losses of virus, the experiments designed to concentrate, purify, and identify the virus failed to reach fruition.

In 1931 a department of plant pathology was established in the Rockefeller Institute for Medical Research near Princeton, New Jersey. Louis O. Kunkel was brought from the Boyce Thompson Institute for Plant Research at Yonkers, New York, to head the new department. Kunkel felt the time was ripe to add a chemist to the team he was organizing to study plant virus diseases. At this time, Wendell Meredith Stanley (Figure 1), a young organic chemist who had received his doctorate under the tutelage of Roger Adams at the University of Illinois, was working with the noted cell physiologist, W. J. V. Osterhout, at the New York branch of the Rockefeller Institute for Medical Research. Stanley was persuaded to join the Princeton group, and in 1933 began his now-famous studies on TMV.

In preliminary experiments, Stanley worked through previous methods of purification and modified them, especially with respect to the pH used in various steps. Infectivity was closely followed for the first time in the fractionation procedures by use of Holmes' newly developed method of local lesion assay (Holmes 1929). Stanley also took advantage of the pres-

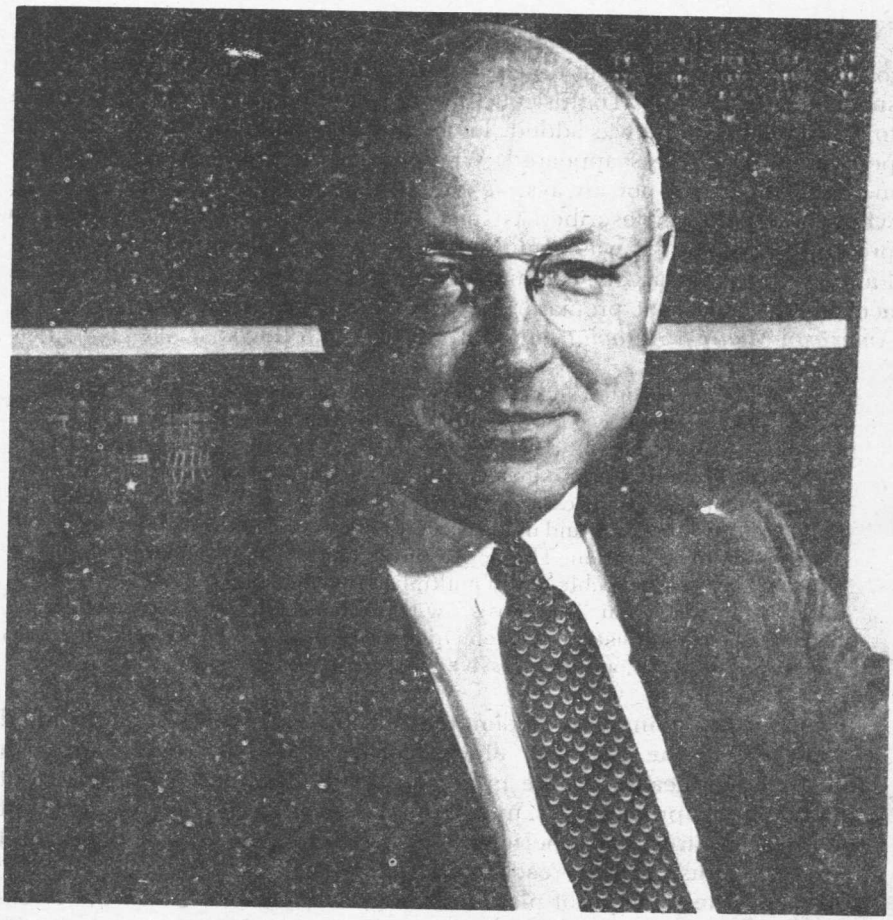


Fig. 1. Wendell Meredith Stanley, 1904–1971.

ence in the Institute of Northrop, Kunitz, Herriott, and Anson, who were engaged in their classic studies on the isolation and properties of crystalline proteolytic enzymes. The proximity of these workers provided, among other things, access to crystalline pepsin, which was used in a crucial experiment of a series on the effect of chemical reagents on viral activity. Stanley (1934b) found that the infectivity of TMV was largely destroyed by pepsin at a pH at which the virus was stable when pepsin was omitted. This result led Stanley (1934) to state, "It seems difficult to avoid the conclusion that tobacco mosaic virus is a protein, or closely associated with a protein, which may be hydrolyzed with pepsin."

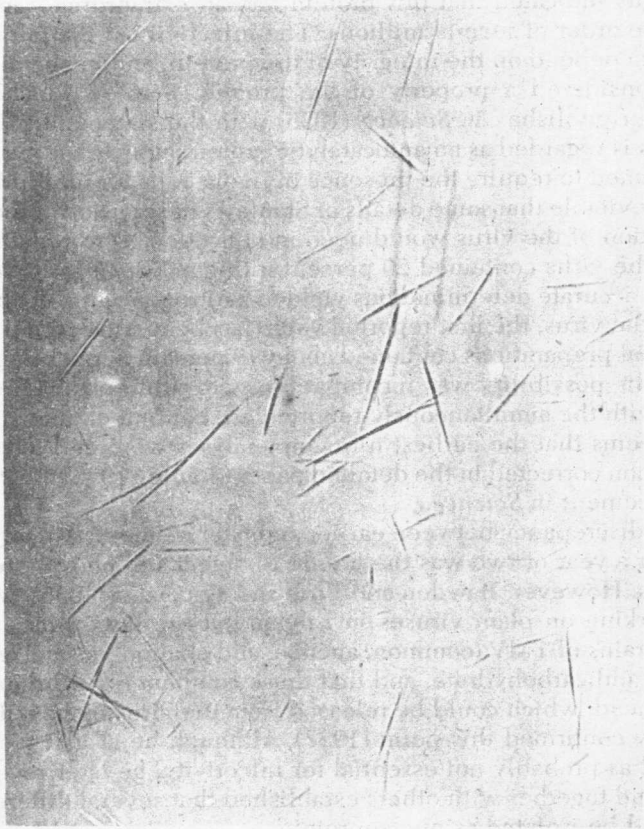


Fig. 2. Crystals of tobacco mosaic virus.

Proceeding, then, with the methods of a protein chemist, Stanley combined repeated precipitation with ammonium sulfate with decolorization by treatment with lead subacetate to obtain high yields of purified virus. Such virus in aqueous solution was crystallized by adding sufficient saturated ammonium sulfate to cause turbidity; then with stirring, adding slowly 0.5 saturated ammonium sulfate in 5 percent acetic acid. Needlelike crystals like those shown in Figure 2 were thus obtained. Such crystals when dissolved were infectious at dilutions as high as 10^9, and the infectivity of the material, in contrast to that of Vinson's preparations, was not lost by as many as ten successive recrystallizations.

From the results of many different tests, the crystalline material appeared to be protein, and preliminary osmotic pressure and diffusion

measurements indicated that this protein had an extraordinary molecular weight of the order of several millions. The infectivity of the preparations was shown to depend on the integrity of the protein, and hence infectivity could be considered a property of the protein. Stanley concluded his historic paper published in *Science* (1935) with the statement: "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."

It was inevitable that some details of Stanley's description of the chemical constitution of the virus would need modification. One was the initial report that the virus contained 20 percent nitrogen. Since his own subsequent, more accurate determinations yielded a nitrogen value of about 16.6 percent for the virus, the first reported value has been interpreted to mean that the initial preparations contained about 70 percent ammonium sulfate. However, this possibility was incompatible with other observations, and especially with the simultaneously reported ash content of only 1 percent. Hence, it seems that the earliest nitrogen analyses were faulty, but these were very soon corrected in the detailed paper (Stanley 1936) that followed the announcement in *Science*.

Another discrepancy between earlier and later elementary analyses that persisted for a year or two was the failure to detect any phosphorus in the preparations. However, Bawden and Pirie and associates (1936), who were actively working on plant viruses in England at the same time, reported that three strains of TMV (common, aucuba, and enation mosaic) contained phosphorus and carbohydrate, and that these components were present in ribonucleic acid, which could be released from the virus by heat denaturation. Stanley confirmed this point (1937). Although he at first viewed the nucleic acid as probably not essential for infectivity, he later reversed his judgment, and together with others established that several different plant viruses could be isolated as nucleoproteins.

In this connection, the earlier analyses of a bacterial virus by Max Schlesinger, working at the Institut für Kolloidforschung in Frankfurt, Germany, tend to be overlooked, probably because of the more extensive and definitive studies on TMV. However, Schlesinger (1934) found that a phage preparation that gave strong color reactions for protein and yet gave a negative test for bacterial antigen contained about 3.7 percent phosphorus. This led him to suggest that nucleoprotein might be a major component of bacteriophages, but the proposal lacked the force it would have carried had the presence of purine and pyrimidine bases been demonstrated.

Thus, the chemical era of virology was launched. The impact on research of Stanley's findings was aptly summarized by a pioneer animal virologist, Thomas M. Rivers, when he presented Stanley to receive the gold medal of the American Institute of the City of New York in 1941 (Rivers 1941). His remarks, in part, were as follows:

Stanley's findings, which have been confirmed, are extremely important because they have induced a number of investigators in the field of infectious diseases to forsake old ruts and seek new roads to adventure. As much as many bacteriologists hate to admit it, Stanley's proof that tobacco mosaic virus is a chemical agent instead of a microorganism is certainly very impressive. . . . In fact, the results of Stanley's work had the effect of demolishing bombshells on the fortress which Koch and his followers so carefully built to protect the idea that all infectious maladies are caused by living microorganisms or their toxins. In addition, his findings exasperate biologists who hold that multiplication or reproduction is an attribute only of life. In the midst of the wreckage and confusion, Stanley, as well as others, finds himself unable at the present time to decide whether the crystalline tobacco mosaic virus is composed of inanimate material or living molecules. In fun it has been said that we do not know whether to speak of the unit of this infectious agent as an "organule" or a "molecism."

II.

Purification of Viruses

A. Some General Principles

Each virus poses an individual purification problem that is related to the properties of the virus, the nature of the host, and the culture conditions. Consequently, it is not possible to outline a purification procedure that will work with equal effectiveness for all viruses. Nevertheless, it is possible to describe a few methods and their underlying principles that have led to purified preparations of some viruses, and, hence, that are potentially useful, separately or in combination, for the purification of other viruses. Attention is directed here to comprehensive reviews on the purification of plant and animal viruses (Steere 1959; Sharp 1953; Maramorosch and Koprowski 1967; Habel and Salzman 1969; Kado and Agrawal 1972).

Methods based on centrifugation have come to dominate the techniques of isolating and purifying viruses as well as to characterize viruses, at least in part. When centrifugation is coupled with a variety of other techniques based on different principles, its potential for purification is greatly enhanced. Some of the methods used as adjuncts to centrifugation include precipitation, adsorption, treatment with enzymes, extraction with organic solvents, treatment with antiserum, electrophoresis, and chromatography.

Two basic facts underlie the purification of viruses by whatever method used: (1) all presently known viruses contain substantial quantities of protein and hence are more or less susceptible to protein fractionating techniques; and (2) the sizes and densities of viruses are such that they are not readily sedimented in low gravitational fields, but are generally sedimentable in characteristic ways in high-speed centrifuges at 40,000 *g* or more.

Some general considerations should also be mentioned here. To determine the effectiveness of any purification procedure, it is essential that a suitable quantitative test for virus infectivity be available. For example, if a virus assay is subject to 50 percent variations (which is not uncommon in biological tests), it is difficult to determine in which fraction the virus is contained or the extent to which the purification conditions are destroying virus activity. Thus, an important contributing factor leading to the discov-

ery of the nature of tobacco mosaic virus was the timely development of a local-lesion assay method (Holmes 1929). With this method the infectivities of fractions could be determined with an error of about 10 percent, a value several times as good as that usually achieved by the older dilution-endpoint assay. Later, assays of bacterial and animal viruses were developed that resembled the plant virus assays in the sense that at appropriate concentrations of virus a linear relationship was observed between concentration of virus and numbers of colonies of virus apparent in tests (local lesions on plant leaves in the case of plant viruses and cell plaques for bacterial and animal viruses). Such assays are illustrated in Figure 3.

If a satisfactory measure of virus activity is available, then it is possible to adjust purification conditions to allow for such factors as pH and thermal stabilities of the virus and salt effects. Lacking information on these factors, it is well to begin by working around neutrality and in the cold. Also, the use of 0.01–0.1 M phosphate buffer has proved a good salt medium for several viruses. Salt mixtures such as Ringer's solution are needlessly complex for most viruses; on the other hand, unbuffered "physiological" saline is deleterious to some viruses owing to its tendency to be somewhat acidic in reaction.

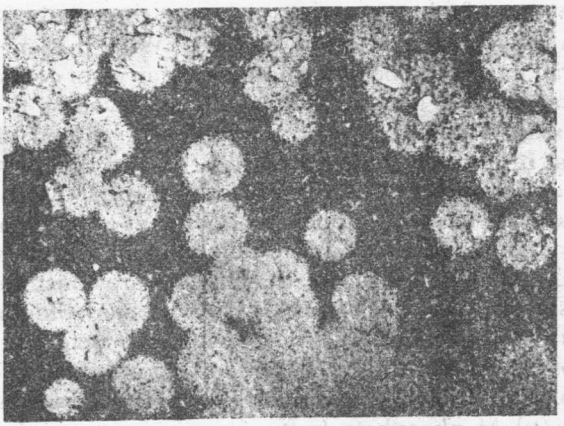
Organic buffers have proved superior to inorganic buffers in some biological systems including viral systems. Thus various salts of tris(hydroxymethyl)aminomethane and organic or inorganic acids provide the socalled Tris buffers with a buffering range between pH 7 and 9. Tris buffers, which have been widely used, do not precipitate divalent cations as phosphate buffers may. However, many biological reactions occur optimally between pH 6 and pH 8 and Tris buffers have poor buffering capacity below pH 7.5; moreover, Tris has a reactive primary amine group that can engage in undesirable or even inhibitory reactions. Consequently, considerable use of a series of zwitterionic buffers (Good et al. 1966) has developed. These buffers are mainly amino acid derivatives, many being N-substituted glycines or N-substituted taurines. They were shown to be superior to Tris or phosphate buffers in several important biological reactions (Good et al. 1966). Some commercially available zwitterionic buffers are listed in Table 1.

1. Centrifugation

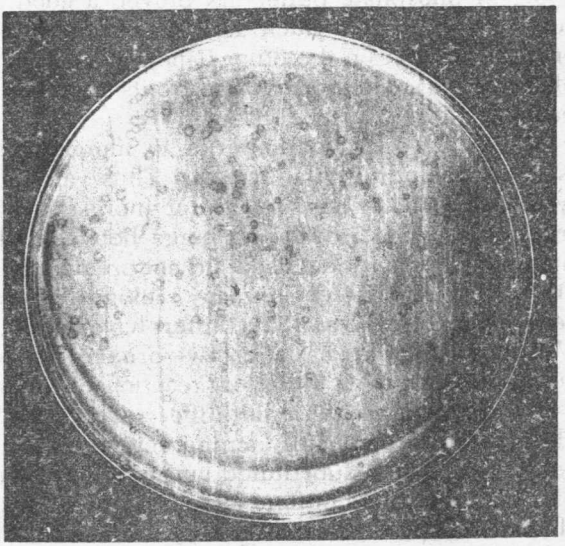
a. Differential Centrifugation

The sizes of most presently known viruses (10–300 nm in diameter) and their densities are such that the viruses are sedimented from solution in an hour or two in centrifugal fields of 40,000–100,000 $\times$ *g*. Such centrifugal fields were achieved in the early years of virus purification with air-driven

(a)



(b)



(c)