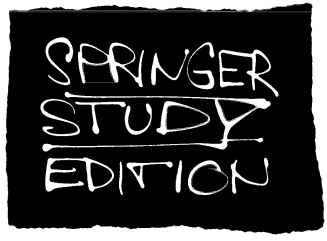
CHEMISTRY OF VIRUSES


SPRINGER
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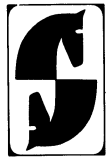
EDITION

CHEMISTRY OF VIRUSES

Second Edition

C. A. Knight
Department of Molecular Biology and the Virus Laboratory
University of California, Berkeley



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This book is affectionately dedicated to Billie, Tom, Susie, and Bob.

Claude A. Knight
Department of Molecular Biology
University of California
Wendell M. Stanley Hall
Berkeley, California 94720

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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-

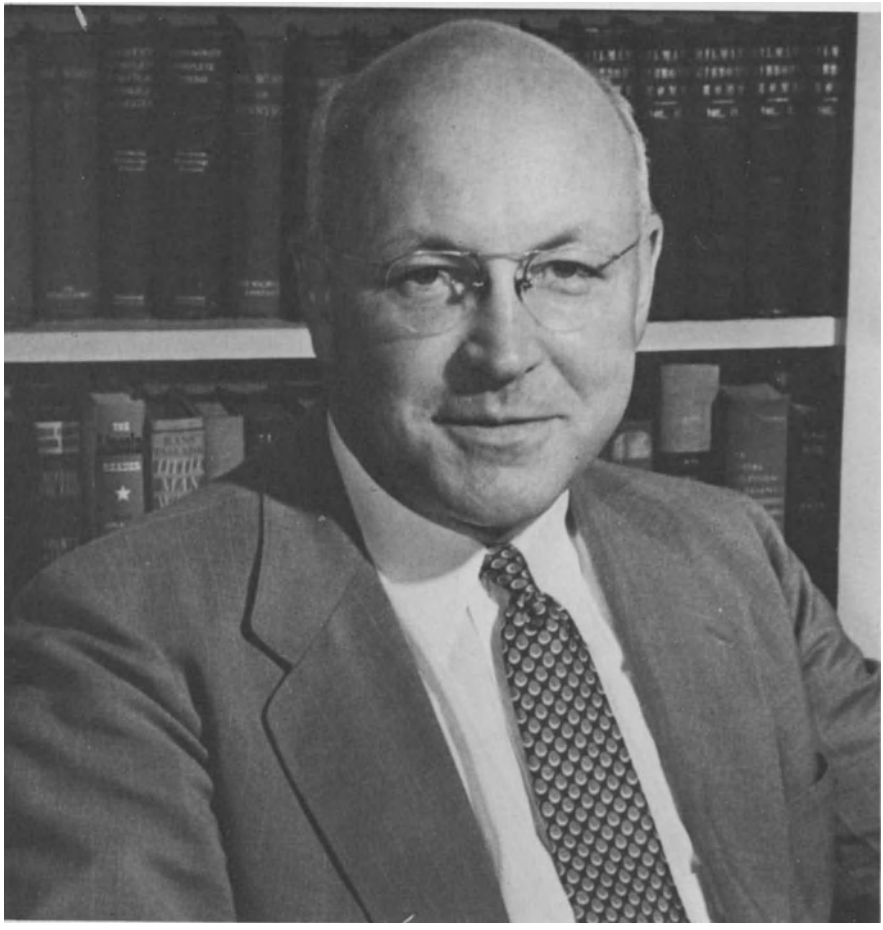


Fig. 1. Wendell Meredith Stanley, 1904–1971.

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."

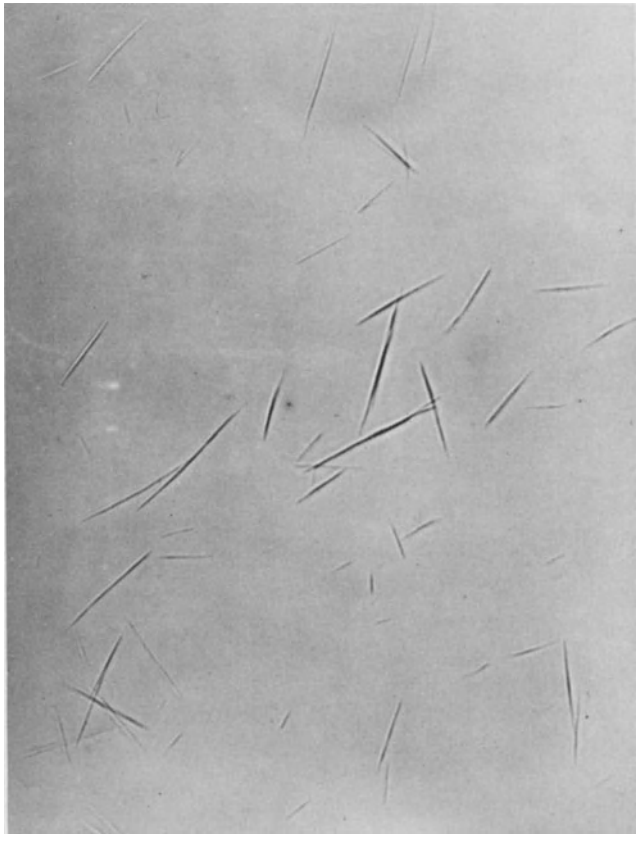


Fig. 2. Crystals of tobacco mosaic virus.

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 "molechism."

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.

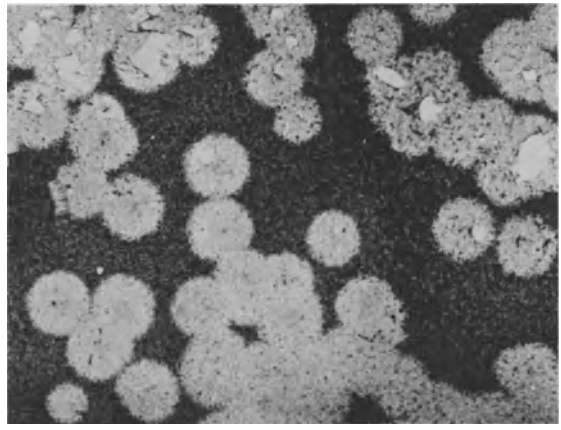
Organic buffers have proved superior to inorganic buffers in some biological systems including viral systems. Thus various salts of tris(hydroxymethyl)aminomethane and organic or inorganic acids provide the socalled Tris buffers with a buffering range between pH 7 and 9. Tris buffers, which have been 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 × *g*. Such centrifugal fields were achieved in the early years of virus purification with air-driven

(a)



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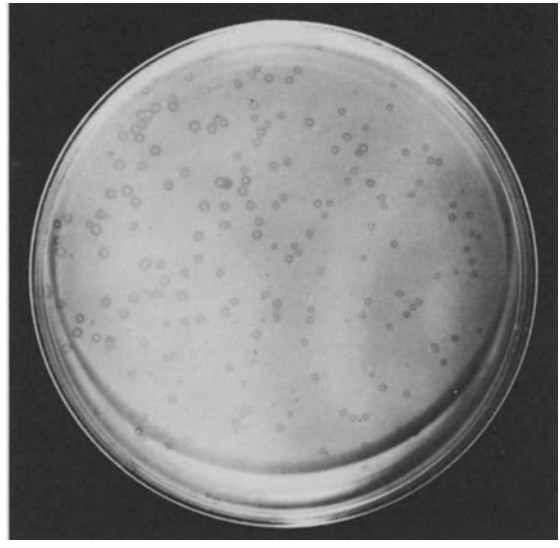

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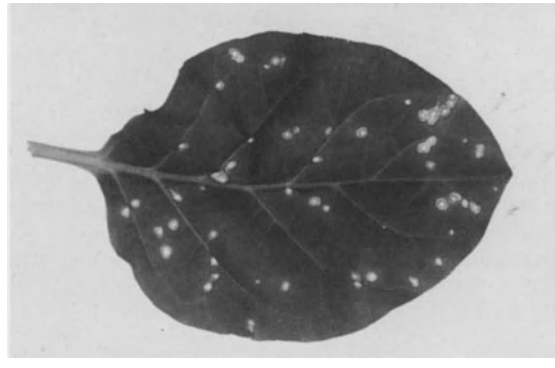


rotors (see Stanley et al. 1959), but these machines have now been largely superseded by electrically driven, commercially available centrifuges.

Some outstanding features of these electrically driven centrifuges are that they are compact, simple in service requirements, and are designed so as to give hours of uninterrupted service with maximum safety to the operator and to the mechanism. The rotors are made of duralumin alloy, a strong substance but relatively light. The same machine accommodates a variety of rotors, most of which hold ten or more plastic tubes in angular holes. The tubes are available in a variety of sizes to match the rotors so that as little as 2 ml or as much as 940 ml can be handled in a run. Both tubes and rotors are sealed, and since the rotor spins in a vacuum, the initial temperatures of rotor and sample change very little during a run. A refrigeration unit around the walls of the vacuum chamber makes it possible to avoid even slight rises in temperature if this is necessary. The force fields obtainable in one commercial model range from 59,000 *g* in the 21,000-rpm rotor, which holds 940 ml, to 198,000 *g* in the 50,000-rpm rotor, which holds 100 ml.

A cardinal feature of the electrically driven centrifuge, aside from its high-speed motor, is the flexible shaft linking rotor with motor. This allows some inexactness in balance of tubes placed opposite one another, for the rotor seeks its own axis of rotation on the flexible shaft.

Some viruses can be obtained in highly purified preparations using only the machine just described. The process of differential centrifugation means simply the application of alternate cycles of low-speed and high-speed centrifugation. This can be done in the same centrifuge, although, commonly, a separate and simpler angle centrifuge is used for the low-speed cycles. The isolation and purification of tobacco mosaic virus by differential centrifugation may be described as follows:

1. Frozen, infected tobacco plants are ground in a meat chopper, 3

Fig. 3. Plaques and local lesions representing infection by three major classes of viruses. Each plaque or spot illustrated represents several hundred cells although each is thought to have originated from the infection of a single cell.

a. Plaques caused by vesicular stomatitis virus in a monolayer of chick fibroblast cells attached to one side of a prescription bottle. After culture under a layer of agar, the overlay is removed and the sheet of cells is fixed and stained with an alcoholic crystal violet solution, rinsed, and air-dried. The background of uninfected cells retains stain, whereas groups of infected cells, which are grossly degraded by the infection, seem clear. (Courtesy F. L. Schaffer.)

b. Plaques caused by phage P22 on a lawn of *Salmonella typhimurium* bacteria. The bacterial lawn is on nutrient agar in a petri dish. The plaques represent groups of lysed cells. (Courtesy J. R. Roth.)

c. Local lesions (necrotic spots) on a leaf of tobacco (*Nicotiana tabacum* L. cv. Xanthi nc) caused by infection with tobacco mosaic virus. Each necrotic spot represents a group of dead cells and the virus is confined to such lesions.

Table 1. Some Zwitterionic Buffers[a]

Name		Useful pH Range[b]	Molarity of Saturated Solution at 0°	Binding of Ca^{2+} and Mg^{2+}	
Common	Chemical				
ACES	N-(2-acetamido)-2-aminoethane sulfonic acid	6.0–7.5	0.2	+	+
ADA	N-(2-acetamido)-2-iminodiacetic acid	5.8–7.4	2.5	+	+
BES	N,N-bis(2-hydroxyethyl)-2-aminoethane sulfonic acid	6.4–7.9	3.2	–	–
Bicine	N,N-bis(2-hydroxyethyl) glycine	7.7–9.1	1.1	+	+
Bis-Tris Propane	1,3-bis(tris[hydroxymethyl]-methylamino)-propane	6.0–10.0			
CAPS	Cyclohexylaminopropane sulfonic acid	9.7–11.1			
EPPS	4-(2-hydroxyethyl)-1-piperazine propane sulfonic acid	7.4–8.6			

HEPES	N-2-hydroxyethylpiperazine N′-2-ethanesulfonic acid	6.8–8.2	2.2	–	–
MES	2-(N-morpholino) ethane sulfonic acid	5.5–7.0	0.6	+	+
MOPS	Morpholinopropane sulfonic acid	6.5–7.9			
PIPES	Piperazine-N,N′-bis [2-ethanesulfonic acid]	6.1–7.5		–	–
TAPS	Tris (hydroxymethyl) methylaminopropane sulfonic acid	7.7–9.1			
TES	N-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid	6.8–8.2	2.6	–	–
Tricine	N-tris (hydroxymethyl)-methyl glycine	7.4–8.8	0.8	+	+

[a]Compiled from Good et al. 1966 and from the March 1974 catalog Sigma Chemical Co., St. Louis, MO 63178, U.S.A.

[b]The pKa values at 20° for these compounds lie approximately midway in the useful range.

percent by weight of dipotassium phosphate is added to the mash, and the mixture is thawed with occasional stirring.

2. The juice is separated from the plant pulp in a basket centrifuge or with a press.

3. The expressed juice is clarified by centrifuging for 10 min in an angle centrifuge at about 8,000 rpm (6,000–8,000 *g*). The angle pellet is discarded (this contains starch, pigmented material, denatured protein, and so on).

4. The clarified juice is centrifuged at 21,000 rpm (about 59,000 *g*) for 1 hr. The supernatant fluid is discarded and the virus pellet is covered with 0.1 M phosphate buffer at pH 7 and allowed to soak overnight at 4°. The softened pellets dissolve readily with a little stirring such as that produced by squirting the liquid up and down with a dropping pipet.

5. The virus solution is centrifuged at low speed as in step 3 and the pellet (pigmented material, denatured protein, and so on) discarded.

6. The supernatant fluid is centrifuged at 21,000–40,000 rpm for 1 hr. (The virus becomes increasingly difficult to sediment as it is concentrated, particularly if the salt concentration is lowered.) The supernatant fluid is discarded and the pellets are dissolved again in 0.1 M phosphate buffer at pH 7, and centrifuged at low speed.

7. The alternate low-speed, high-speed runs are continued for four complete cycles. If the virus is to be lyophilized, the last two cycles are made in distilled water, thus removing salt. Although it is probably not necessary with TMV and some of the more stable viruses, refrigeration is employed in the high-speed centrifugation and the material is kept cold throughout the preparative procedure.

A technique for preparative microcentrifugation of viruses and other entities of similar size has been described by Backus and Williams (1953). In this method, pellets obtained with conventional centrifugation equipment are resuspended in 0.01–0.1 ml of diluent and are then transferred and sealed into "field-aligning" glass or quartz capsules. These are suspended in a solvent of suitable density in a standard plastic centrifuge tube and centrifuged in an angle rotor at an appropriate speed. By using supplementary equipment, such as a spectrophotometer, these capsules can also be used for analytical ultracentrifugation of virus preparations (Backus and Williams 1953).

b. Density-Gradient Centrifugation

A powerful adjunct to the conventional differential centrifugation procedure for the isolation and purification of viruses is density-gradient centrifugation (Brakke 1960, 1967; Vinograd and Hearst 1962; Schumaker 1967). Not only can separations be achieved by this method that are impossible in ordinary sedimentation but under appropriate conditions, densities and sedimentation coefficients also can be estimated.

The essence of the density-gradient system is the separation of particles partly or entirely on the basis of their densities in a convection-free medium. There are many modifications of the method, which, however, differ mainly in operational details such as (1) material used to form the gradient, (2) use of preformed gradient or one formed during the sedimentation, (3) gravitational field, or (4) length of time of centrifugation, especially in relation to equilibrium conditions. The results obtained will depend largely upon these factors.

In practice the different modifications of density-gradient centrifugation may be considered to fall into two classes:

1. Rate-Zonal (or Velocity) Density-Gradient Centrifugation [also termed "gradient differential centrifugation" by Anderson (1955)]. In this procedure the virus solution is layered on top of a preformed gradient, such as a sucrose or glycerol density gradient, and centrifuged in a swinging bucket rotor for 0.5–3 hr at about 70,000–170,000 *g*. (The time required for rate-zonal centrifugation is approximately equivalent to that required to sediment the virus completely in ordinary centrifugation in the same gravitational field.) Particles appear in zones according to their sedimentation rates; hence the term "rate zonal."

While density of the sedimenting particles is a primary factor in determining the zones obtained, size and shape of the particles and viscosity of the medium are also involved in these nonequilibrium conditions. Thus in rate-zonal centrifugation, virus particles tend to concentrate in a zone in which, barring interaction, contamination is mainly restricted to particles whose size, shape, and density combine to give them about the same sedimentation velocity as the virus.

An example of the use of the popular rate-zonal method of density-gradient centrifugation is found in the purification of poliovirus by Schwerdt and Schaffer (1956), and may be summarized as follows:

1. Density gradients were set up in 5-ml cellulose acetate tubes by layering 0.7-ml vol of 45, 37, 29, 21, and 11 percent (by weight) sucrose solutions in 0.14 M NaCl. A continuous gradient was established by allowing the tubes to stand at 4° for 12 hr.

2. About 0.7 ml of partially purified poliovirus (butanol extracted, enzyme treated, and two times ultracentrifuged) was layered on top of each density gradient and centrifuged in a swinging bucket rotor at 30,000 rpm (about 70,000 *g*) for 2 hr.

3. A narrow beam of light from a microscope light was shone down through the gradient column; and when viewed at right angles against a dark background, four bands could be distinguished, which were designated, from top to bottom, A, B, C, D, respectively.

4. Each of the four bands was removed in turn, starting with the upper one, A, by puncturing the tube with a hypodermic needle and withdrawing the appropriate volume of liquid into a syringe.

5. Various tests were made on the material of the four bands (Schwerdt and Schaffer 1956; LeBouvier et al. 1957). Virtually all of the infectivity was found in band D, but particles of similar dimensions and serological properties were also found in band C. The particles in band C, however, contained no more than a few percent RNA, whereas those in band D were found to contain 25–30 percent RNA.

A similar separation of two classes of particles that had essentially the same size and shape but differed in nucleic acid content (and hence in density) was made with partially purified Shope papilloma virus, using rate-zonal centrifugation in sucrose or glycerol density gradients (Williams et al. 1960). In conventional differential centrifugation, the two types of particles (nucleic acid containing and nucleic acid free) occur together, but they are nicely separated on the density-gradient column, and it was demonstrated that only the nucleic acid-charged particles are infectious (Figure 4).

Many examples of the applications of rate-zonal centrifugation to plant virus problems are reviewed by Brakke (1960).

2. *Equilibrium (or Isopycnic) Density-Gradient Centrifugation.* If the rate-zonal procedure is continued for a period of hours, most of the particles reach a zone corresponding to their densities (isopycnic position). Thus the zones obtained are essentially the equilibrium ones with respect to densities of particles.

Commonly, however, the concentration gradient is formed either prior to or during centrifugation (Meselson et al. 1957). Inorganic salts—cesium chloride, rubidium chloride, potassium bromide, and so on—have been employed, usually in the concentration range of about 6–9 M, and the establishment of a gradient depends upon the partial sedimentation of these salts in the centrifugal field. The virus solution is introduced either before or after formation of the gradient, and centrifugation is then continued (12–24 hr) until the particles have reached a point in the suspending medium of equal density (isopycnic position).

The original report of Meselson et al. (1957) nicely illustrates the power of the equilibrium density-gradient centrifugation method. They showed that the normal DNA of T2 bacteriophage could be readily distinguished from T2 DNA in which some of the thymine had been substituted by the denser component, 5-bromouracil.

Some other applications of the equilibrium density-gradient centrifugation method include the purification of ØX174 bacteriophage (Sinsheimer 1959a) and the demonstration that its DNA differs significantly in density from the DNA of the host, *Escherichia coli* (Sinsheimer 1959b); the demonstration of differences in density between strains of tobacco mosaic virus (Siegel and Hudson 1959), and between strains of herpes simplex virus (Roizman and Roane 1961); and the purification of potato virus X (Corbett 1961) and of Rous sarcoma virus (Crawford and Crawford 1961). Also,

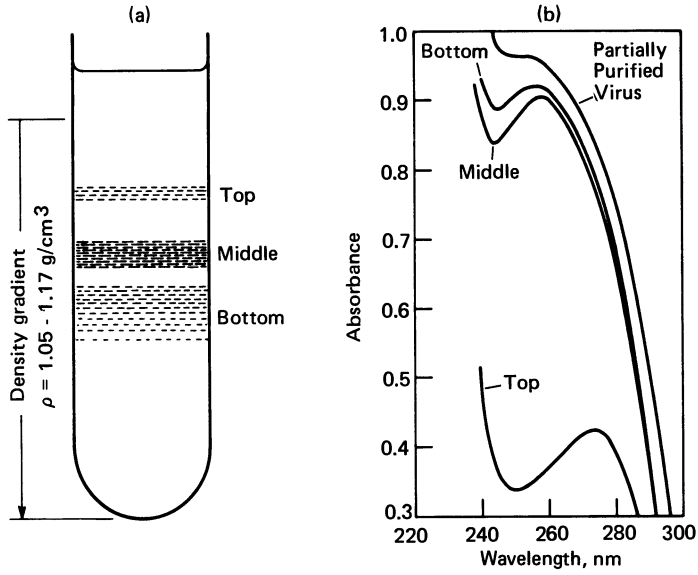


Fig. 4. *a*. Sketch of the bands observed after rate zonal density-gradient centrifugation of partially purified Shope papilloma virus in a glycerol gradient for 2.5 hr at 24,000 rpm in an SW-25 swinging bucket rotor. Material in the top band proved to be very low in DNA, virtually noninfectious, and exhibited particles that appeared hollow by negative staining electron microscopy (see Fig. 33). Material in the middle and bottom bands (bottom material appears to be an aggregate of middle) had much DNA, was highly infectious, and contained particles that appeared filled (dense) in negative staining electron microscopy (see Fig. 33) (Kass and Knight 1965).

b. Ultraviolet absorption curves of partially purified Shope papilloma virus and of fractions obtained from rate zonal centrifugation as sketched in 4*a*. Note that the peak of absorbance of top material is near 280 nm, characteristic of proteins, whereas the peak absorbances of middle and bottom components are near 260 nm, characteristic of nucleic acid and nucleoproteins having more than 5 percent nucleic acid (Williams et al. 1960).

differences in density between standard and "incomplete" influenza viruses have been shown by equilibrium sedimentation in cesium chloride (Barry 1960). Since this technique is almost universally employed in virology, this listing is far from complete.

There has been some concern about the possible deleterious effect of the gradient materials on viruses, especially in the equilibrium method, but thus far most viruses have appeared quite stable in strong cesium chloride and similar salts. While only a milligram or less of material is normally applied per tube for density-gradient sedimentation, it is possible to greatly expand the quantity of material separated by this technique if suitable equipment is employed (see Anderson and Cline 1967).

2. *Enzymatic Treatment*

A property that has aided in the purification of some viruses is the resistance of most of them, unless denatured, to attack by proteolytic enzymes and nucleases. For example, Bawden (1950) and co-workers used snail enzymes to effect a greater release of plant viruses from leaf tissues, or trypsin and chymotrypsin to digest pigment-protein complexes attached to plant viruses. Likewise, Bachrach and Schwerdt (1954) and Schwerdt and Schaffer (1955, 1956) used pepsin and nucleases in purifying poliomyelitis virus. Herriott and Barlow (1952) used treatment with deoxyribonuclease as a step in the purification of T2 bacteriophage in order to destroy host DNA, which might otherwise adsorb to the phage.

3. *Extraction with Organic Solvents*

The purification of poliomyelitis virus is aided by an extraction with *n*-butanol (Bachrach and Schwerdt 1952, 1954). In this procedure, the virus remains in solution in the aqueous layer; lipids, when present, are extracted into the butanol layer, and a significant proportion of the nonviral proteins are denatured and appear mainly in the interphase. A modification of this technique, employing equal parts of *n*-butanol and chloroform, has been used successfully in the purification of some plant viruses (Steere 1956, 1959; Frisch-Niggemeyer and Steere 1961).

Certain fluorocarbons, such as Freon 112 ($FCl_2C\text{-}CCl_2F$) or Genetron 226 ($F_2ClC\text{-}CCl_2F$), alone or mixed with *n*-heptane, have been widely employed in the purification of animal viruses (see Gessler et al. 1956a, 1956b; Philipson 1967b; Brown and Cartwright 1960 for examples and other references). Treatment with the fluorocarbon genesolv-D was an important step in the purification of cytoplasmic polyhedrosis virus of the silkworm (Lewandowski et al. 1969). The fluorocarbon extraction procedure was also used with some success in the isolation and purification of tobacco mosaic and ringspot viruses, but caused loss of infectivity when applied to the isolation of common cucumber mosaic virus (Porter 1956). The success of the fluorocarbon treatment of virus-containing tissue homogenates appears to lie in the efficiency of fluorocarbon emulsions in gathering and holding nonviral proteins and lipids in the organic phase while concentrating the viruses, without significant loss of infectivity, in the aqueous phase.

4. *Precipitation Methods*

An outline of a precipitation method, adapted from Stanley's procedure (1938), used to purify tobacco mosaic virus illustrates some of the details and principles of this method:

1. Infected plants are harvested and frozen. (Many viruses will withstand freezing and thawing in tissues or in crude extracts, whereas normal cellular components are frequently denatured. Hence this step may aid in purification.)

2. The frozen plants are minced in a meat chopper, and 3 percent by weight K_2HPO_4 (in a 50 percent solution) is stirred into the mash, which is then allowed to thaw. The buffer serves a dual purpose: aiding in the extraction of virus and maintaining a suitable pH. (Finer grinding, such as obtained in a roller mill [Bawden 1950] will, in some instances, release considerably more virus than a meat chopper. However, it is often more difficult to purify the virus from such mashes and there is evidence for greater destruction of virus in fine grinding.)

3. The juice is separated from the solids by allowing the mash to drain in the cold through several thicknesses of gauze or cheesecloth followed by further expression of juice from the mash in a canvas bag in a laboratory press. Alternatively, the juice may be separated from the solids by centrifuging the mash in a basket centrifuge.

4. The juice is clarified by passing through about 1 cm of diatomaceous silica (Celite filter-aid) on a Buchner funnel.

5. The virus is precipitated from the clarified juice by addition of 30 percent by weight of ammonium sulfate, and is separated by filtration on a thin layer of Celite.

6. The virus is redissolved by suspending the Celite in 0.1 M phosphate buffer at pH 7 and is separated from the Celite by filtering on a Buchner funnel.

7. The virus is precipitated again by addition of about 11 percent by weight of ammonium sulfate and filtered again on Celite. (Less ammonium sulfate is required to precipitate the virus from purified solutions than from crude juice.)

8. After two or three more precipitations with ammonium sulfate, the last precipitate is dissolved in water and the solution adjusted to pH 4.5, causing precipitation of the virus. (The virus is insoluble at its isoelectric point and for a considerable zone on either side depending on salt concentration.)

9. The virus is filtered on Celite as above and dissolved in water at pH 7. This constitutes the purified preparation of virus. If it is desired to crystallize the virus, saturated ammonium sulfate is added dropwise to the aqueous solution until a cloudiness develops; then 5 percent acetic acid in half-saturated ammonium sulfate is added with vigorous stirring until a lustrous sheen signals the presence of needlelike crystals.

Precipitation with ammonium sulfate has also been used to obtain highly purified crystalline preparations of tomato bushy stunt, tobacco necrosis, southern bean mosaic, turnip yellow mosaic, and squash mosaic viruses (Bawden 1950; Price 1946; Markham et al. 1948; Takahashi 1948).

In some cases magnesium sulfate is substituted for the ammonium salt, and occasionally ethanol is used, either to precipitate the virus or, by selection of proper conditions, to precipitate impurities from the virus solution.

Precipitation by neutralized ammonium sulfate has been helpful in concentrating myxo- and paramyxoviruses. How this step fits into the procedure for purifying simian virus 5, Newcastle disease virus, and Sendai virus is illustrated in the following example (Mountcastle et al. 1971).

1. All three viruses were grown in monolayer cultures of bovine kidney cells on plastic surfaces in reinforced Eagle's medium. The culture medium into which virus was released from the infected cells was harvested 24–42 hr after inoculating the cells.
2. Cell debris was removed by sedimentation at 3,000 *g* for 20 min and virus was precipitated from the supernatant by addition of an equal volume of saturated ammonium sulfate.
3. The precipitate of crude virus was resuspended in Eagle's medium and banded twice in linear 15–40 percent (w/w) potassium tartrate density gradients at 23,000 rpm for 2.5 hr in a Spinco SW 25.1 rotor.
4. The viral band in each case was dialyzed overnight against Eagle's medium or distilled water, and either used immediately or stored at −60°C.

Lowering the pH of virus extracts may precipitate the virus, resulting in concentration and some purification. However, caution is needed in such precipitations because of the sensitivity of some viruses to acidic conditions. Even pH 5 is destructive to many strains of influenza virus. On the other hand, some plant viruses are both stable and soluble at pH 5, whereas host cell constituents are not. Thus, white clover mosaic virus can be purified by adjusting the pH of centrifugally clarified plant sap to 5 with acetic acid and then discarding the resultant precipitate (Miki and Knight 1967). Virus is recovered after this treatment by a few cycles of differential centrifugation.

Precipitation techniques have frequently been used with animal viruses to quickly concentrate virus from dilute solution. For example, the virus in large volumes of allantoic fluid from chick embryos infected with influenza virus was concentrated and partially purified by precipitation in the cold with 25–35 percent methanol (Cox et al. 1947). This procedure has also been applied to the concentration and partial purification of eastern equine encephalomyelitis, MEF poliomyelitis, mumps, and Newcastle disease viruses (Pollard et al. 1949).

Protamine is another precipitating agent used in the concentration and partial purification of animal viruses (Sharp 1953). In some cases this reagent precipitates the virus more or less selectively, whereas in other instances contaminating materials are thrown down and the virus largely remains in the supernatant fluid.

Commercial yeast nucleic acid was found useful in enhancing the acid precipitation of poliovirus (Charney et al. 1961). Previously it had been

shown that poliovirus could be precipitated from tissue culture filtrates by acidifying to about pH 2.5 (Mayer et al. 1957; Charney 1957), but acid treatment alone resulted in inexplicably variable degrees of precipitation of the virus. However, essentially complete and reproducible precipitation of the virus is effected by adding 50 μg of commercial sodium ribonucleate per milliliter of tissue culture fluid (containing 0.1–1 μg virus/ml) before lowering the pH to 2.5. After the virus is concentrated in this manner, other methods, such as differential centrifugation, can be used for further purification.

5. *Adsorption Methods*

Adsorption of viruses on, and subsequent selective elution from, various materials has been used with moderate success to purify a few plant and animal viruses, but this principle has seldom been used alone in the concentration and purification of viruses. Historically, however, adsorption techniques were among the first methods employed for concentrating and purifying animal viruses (Stanley 1938), and, with the development of new adsorbents such as ion exchange resins and cellulose derivatives, this approach has new potential.

A common procedure with adsorbing agents is to cause a precipitate to form in the crude virus suspension, upon which the virus is adsorbed and from which it is subsequently eluted, usually by treatment with an appropriate buffer. Included among adsorbing materials of this sort, which have been used especially with animal viruses, are aluminum phosphate, calcium phosphate, calcium sulfate, and ferric and aluminum hydroxides. In addition, kaolin, charcoal, and alumina have been employed as adsorbing agents. Sometimes impurities are adsorbed rather than the virus. Such an example was observed with potato virus X (Corbett 1961) from whose crude preparations pigments and microsomal material were removed preferentially by activated charcoal, permitting subsequent isolation of nonaggregated virus rods of high infectivity by centrifugation on a density gradient.

"Salting out" chromatography on Celite and on calcium phosphate columns has helped in the purification of chicken tumor virus (Riley 1950), tobacco mosaic virus (Tiselius 1954), herpes virus, encephalomyocarditis virus, and others (Philipson 1967a).

Tobacco mosaic virus and related materials, potato virus X, southern bean mosaic virus, rice dwarf virus, and internal cork virus of sweet potatoes have been purified with moderate success on ecteolacellulose, DEAE cellulose, carboxymethyl cellulose, chitin columns, and agar gel columns (von Tavel 1959; Levin 1958; Commoner et al. 1956; Cochran et al. 1957; Townsley 1961; Steere and Ackers 1962; Venekamp 1972). At present, none of these procedures seems likely to replace centrifugation for

the routine purification of TMV or other viruses, although they do offer advantages for certain specific objectives.

Similarly, influenza, adenovirus, and T2 phage have been purified on cellulose columns of one sort or another (Laver 1961; Haruna et al. 1961; Taussig and Creaser 1957), and several different animal viruses have been partially purified by adsorption on and elution from ion exchange resins (Lo Grippo 1950; Muller 1950; Muller and Rose 1952; Takemoto 1954; Matheka and Armbruster 1956a, 1956b; Philipson 1967a).

Outstanding success was reported in removing tenacious pigment from southern bean mosaic virus with an ion exchange column (Shainoff and Lauffer 1956, 1957). In one experiment, 490 mg of 520 mg virus applied were recovered when the column was eluted with chloride-phosphate at room temperature at a rate of 100 ml/hr. The pigment could be seen as a brownish discoloration of the resin along two-thirds the length of the column.

Incidentally, the problem of tenacious pigments is rather common to the preparation of plant viruses and the following approaches have been used in attempts to solve it: (1) displacement of the pigment by polyvalent anions (Ginoza et al. 1954); (2) extraction with organic solvents (Steere 1959); (3) treatment with proteolytic enzymes (Bawden 1950; Markham and Smith 1949); (4) column chromatography (Shainoff and Lauffer 1956, 1957); (5) treatment with charcoal (Corbett 1961); and electrophoresis (Goldstein et al. 1967).

The use of magnesium pyrophosphate for the rapid purification of coliphages (Schito 1966) and aluminum phosphate for the purification of influenza viruses (see below) are other successful applications of adsorption techniques. Miller and Schlesinger (1955) used aluminum phosphate-silica gel either in a batch-type process or in a column to adsorb influenza virus directly from infectious allantoic fluid. By adapting this column procedure and by using virus preparations that had already been partially purified by differential centrifugation, Frommhagen and Knight (1959) were able to obtain not only highly purified virus, but also specific host material. This host material is important because it is serologically closely related to one or more components inseparably bound to influenza virus particles (Knight 1944, 1946a).

The following outline describes the use of such a column:

1. A glass column about 5 × 100 cm is charged with aluminum phosphate-silica gel, prepared as described by Miller and Schlesinger (1955).

2. Partially purified influenza virus, obtained from infectious allantoic fluid by one cycle of centrifugation (see section on centrifugation), is diluted with 0.125 M phosphate buffer at pH 6.0 (for an A strain like PR8) or with water (for a B strain like Lee) to a concentration containing 100 chick cell agglutinating units (CCA units) per milliliter. This solution is put

through the column (adsorbing cycle) at a rate of about 4 ml/min. The effluent of the adsorbing cycle emerges with a milky opalescence, possesses almost no hemagglutinating power or infectivity, and can be shown by serological tests to be very similar to normal allantoic material. The effluent can be further purified by repeated passage through the column, and can be concentrated by sedimenting at 44,000 g for 20 min. The virus is eluted from the aluminum phosphate by passing through at about 5 ml/min a volume of 0.25 M phosphate buffer at pH 8.0 equal to one and a half times the volume of solution applied to the column. The eluted virus can be concentrated, if desired, by sedimenting it from the eluate at 44,000 *g* for 20 min.

The major disadvantages of the adsorption methods mentioned above are that they are usually not sufficiently selective, and the high salt concentrations generally required for elution may be deleterious to the virus, or in any case, often constitute an unwanted component that can complicate subsequent procedures with the virus. However, as illustrated above, adsorption methods, combined with centrifugation or other techniques, may aid substantially in the purification of viruses.

A special application of adsorption is that which involves viruses and red cells. Several animal viruses have been found to adsorb on and cause agglutination of appropriate erythrocytes, and some of these viruses, though not all, can be readily eluted from the red cells (Hirst 1959; Rosen 1964). The specificity of the adsorption in the case of influenza is so great that this phenomenon was an invaluable step in the preparation of highly purified virus from infective chick embryo fluids and from lung tissue homogenates (Knight 1946b), but density-gradient centrifugation has now largely replaced it. In practice, adsorption on and elution from erythrocytes is accompanied by centrifugation procedures, and it is particularly important to subject eluted virus to two cycles of high- and low-speed centrifugation to free it from red cell proteins (mainly hemoglobin) that may enter the preparation during the adsorption-elution process.

6. *Serological Methods*

Occasionally, it is possible to purify partially a virus by serological methods. This technique was successfully applied by Cohen and Arbogast (1950) to some preparations of bacteriophage. These were treated with antiserum to *Escherichia coli*, causing a precipitate of the traces of cellular debris present. This precipitate was removed by centrifugation and discarded, and the virus was freed of serum elements by two cycles of differential centrifugation.

Likewise, antisera to proteins of healthy plants have been employed to help purify certain plant viruses. The problem of adding serum protein contaminants to the virus under purification applies here as it does with

phages. However, contamination with serum elements can be minimized by use of partially purified antibodies, that is, the gamma globulin fraction, rather than whole serum. After the complex of plant proteins and their antibodies is precipitated, the virus is separated from excess serum globulin by differential or density-gradient centrifugation. Such a technique was successfully applied by Gold (1961) to the purification of a strain of tobacco necrosis virus and subsequently by Fulton (1967a, 1967b) in the purification of rose mosaic, tulare apple mosaic, and tobacco streak viruses.

7. *Electrophoresis*

Conventional solution electrophoresis has been employed in the purification of southern bean mosaic virus (Lauffer and Price 1947), tobacco ringspot virus (Desjardins et al. 1953), tomato ringspot virus (Senseney et al. 1954; Kahn et al. 1955), influenza virus (Miller et al. 1944), blue-green algal virus LPP-1 (Goldstein et al. 1967), among others. However, this method is limited, largely because of the complexity of the equipment required and the difficulties in sampling. Sampling is facilitated in at least one commercial apparatus with a sampling needle that can be seen and controlled by the operator while looking at the migrating boundaries in the optical system (see van Regenmortel 1972).

Convection and sampling problems are greatly reduced in zone elec trophoresis carried out in sucrose density gradients. A procedure of this sort was first used by Brakke (1955) in the purification of potato yellow-dwarf virus, and subsequently was greatly extended by van Regenmortel (1964, 1966, 1972), who used it successfully for separating 20 different plant viruses from contaminating antigenic plant proteins. Several animal viruses have been found to migrate distinctive distances in zone electrophoresis (Polson and Russell 1967), and it is probable that some purification occurs during the process, although this has not been adequately evaluated.

Electrophoresis in solid media such as agar, agarose, or polyacrylamide gels has been effective in the purification of tobacco mosaic virus, potato virus X, and turnip yellow mosaic virus, as well as in the separation of some animal viruses (Townsley 1959; Tiselius et al. 1965; Polson and Russell 1967). However, other purification techniques appear to be more convenient or selective or both.

8. *Partition in Liquid Two-Phase Systems*

Albertsson and co-workers (see Albertsson 1960, 1971) described several two-phase systems of water-soluble polymers, such as dextran-methylcellulose or dextran-polyethylene glycol, in which animal, bacterial, and plant viruses could be selectively concentrated and at least partially

purified. Concentration of a virus by this method is based on its low partition coefficient in the systems used, which by proper adjustment of phase volumes permits concentration of most of the virus in a low-volume phase (or at the interface). In practice, concentrations of 10–100 times were obtained in one step or as much as 100–10,000 times in a two-step operation. Purification occurs mainly because impurities distribute differently from the virus. Proteins, for example, distribute rather evenly (K = 0.5–1). The purification effect is enhanced by repeated partitioning, as, for example, in a countercurrent apparatus. The usefulness of the method is also increased by combination with other techniques such as fluorocarbon extraction (dextran seems to stabilize sensitive viruses in this treatment) or high-speed centrifugation, with or without a density gradient.

The following one-step example (Albertsson 1960) illustrates the possibilities of this technique:

1. ECHO virus, prototype 7, was grown in monkey kidney tissue culture in Parker 199 medium.
2. To 5,000 ml virus culture was added 64 g of a 20 percent (w/w) sodium dextran sulfate solution and 1,390 g of a 30 percent (w/w) polyethylene glycol solution containing 69.5 g NaCl.
3. The mixture was shaken in a separatory funnel and allowed to stand at 4°C for 24 hr for phase separation.
4. Practically all the virus (as judged by infectivity tests) was found in the clear bottom phase in a volume of 50 ml. Hence the virus had been concentrated 100 times. With respect to purification, the original 5,000 ml of virus culture contained 1090 mg nitrogen and the concentrate was found to have only 39.5 mg nitrogen. Thus more than 96 percent of the nitrogen was removed (much of it in the form of particulate material that collected at the interface) with essentially no loss of infectivity.

The advantages claimed for the liquid two-phase separation are mainly its mildness and simplicity. Its strong point appears to be the ease with which rather great concentrations of virus can be achieved. While significant purification of the virus is also obtained, supplemental methods are usually needed when highly purified virus is the objective. There is also the problem of separating the virus from polymer, but this can be done either by repeated ultracentrifugation, which mainly sediments the virus, or by precipitation of the polymer, which, however, usually adds inorganic salt. The virus can be separated from most of the salt by high-speed centrifugation or the salt can be removed by dialysis or gel filtration.

9. *Criteria of Purity*

Prior to chemical analyses, it is essential to evaluate the purity of a virus preparation. A few definitions are required here. Viruses are infectious agents mainly characterized by their small size (10–300 nm in diameter, or

the nonspherical equivalent) and ability to reproduce only in living cells. Traditionally, this is essentially the same as saying that viruses are infectious nucleoproteins or somewhat more complex particles. The traditional view will be followed here despite the suggestion, for which there is considerable justification, that viruses are infectious nucleic acids (Northrop 1961). In these terms, purity means the degree of freedom of viral particles from nonviral components, or, conversely, the extent to which viral particles show gross physicochemical homogeneity. No single test is sufficient to establish this type of purity, but a consistent answer from each of several tests establishes the degree of homogeneity of the preparation in question and hence the reliance to be placed on analytical data and other results obtained with such a preparation.

The degree of homogeneity of a virus preparation with respect to particle size, shape, and density can be evaluated in modern analytical centrifuges (see Stanley et al. 1959; Schramm 1954; Schachman 1959; Markham 1967). Thus a single sedimentation boundary suggests the presence of a single species of particle, two boundaries, two components, and so on.

Furthermore, the nature of the boundary can be significant, for the degree of boundary spreading observed with a homogeneous preparation should be no greater than expected from the diffusion constant, as independently determined. Likewise, the results of the diffusion measurements themselves can provide information regarding the homogeneity of the material. Incidentally, a combination of the results of sedimentation and diffusion measurements permits a calculation of molecular weight, and if supplementary data, such as density or viscosity values, are available, one can estimate the particle radius by application of Stokes' law (Stanley et al. 1959; Schramm 1954; Schachman 1959; Markham 1967).

Another widely applied criterion of purity is electrochemical homogeneity as measured in the electrophoresis apparatus (Stanley et al. 1959; Alberty 1953; Brinton and Lauffer 1959). It can be regarded as good evidence for homogeneity of a virus preparation if the material migrates with a single boundary over the entire pH range within which the virus is stable. This evidence is strengthened if the boundary shows no greater spreading than anticipated from the diffusion constant.

The lower limit of contaminant detectable by either sedimentation analysis or electrophoresis is variable, and is dependent upon the nature of the material and the circumstances of the test. As usually applied in testing virus preparations, these methods cannot be expected to detect less than a few percent of contaminant (Sharp 1953). For many purposes, it is satisfactory to measure purity to this degree, but as the tools for chemical and biological analyses become sharper and sharper, it will be increasingly necessary to remember the limitations of sedimentation and electrophoresis measurements.

The electron microscope can be used to examine directly the physical

homogeneity of a virus preparation. Under favorable conditions it is possible to detect an impurity present in a concentration of as little as 1 percent of the virus (Williams 1954). It is obvious, of course, that impurities will escape detection if they have the same size and shape as the virus particles, or if they are below the size resolved by the microscope. Also, particles present in small number but large in mass are easily overlooked, owing to sampling difficulties (Lauffer 1951). Nevertheless, under favorable conditions it appears that the electron microscope is capable of detecting impurities at a level presently unattainable by any other physical method.

Crystallinity, once considered by many as evidence of purity, has fallen into disrepute (Pirie 1940). This is primarily because of the demonstration that crystalline protein preparations may be contaminated by amorphous material, by crystals of other substances, or by reason of containing mixed crystals, that is, solid solutions. Furthermore, not all proteins or viruses will crystallize regardless of their purity. Nevertheless, it is clear that crystallization usually results in purification, and it seldom occurs unless one constituent is predominant and in a native state. Denatured proteins are known to lose crystallizing ability (Putnam 1953). Therefore, the criticism of crystallinity as a criterion of purity is valid, mainly in the sense that this property does not afford a precise means for determining whether or not any contaminant is present, and, if so, how much. If a virus will crystallize, however, it is still a good preliminary indication of purity.

Frequently, immunochemical methods are used to good advantage in testing the purity of virus preparations (Kabat 1943; van Regenmortel 1966; Bercks et al. 1972). With the use of proper antisera one may detect by means of precipitin or complement fixation tests very small amounts of contaminating tissue antigens. Such impurities were demonstrated in early preparations of tobacco mosaic virus, but the most highly purified preparations, such as are now employed in most chemical studies, give no indication by serological means of the presence of normal antigens, even when tested by the extremely sensitive anaphylactic test (Bawden and Pirie 1937).

Use of the immunochemical approach to test for host antigens in preparations of influenza virus led to the discovery of a previously unfamiliar host-virus relationship. Highly purified preparations of the virus, obtained from allantoic fluid of infected chick embryos, and which were homogeneous in the analytical ultracentrifuge under various conditions and in the electrophoresis apparatus over a wide range of pH, reacted strongly in quantitative precipitin tests with antiserum to material isolated from normal allantoic fluid (Knight 1946a). Similarly, highly purified virus isolated from mouse lungs was found to contain an antigenic component characteristic of normal mouse lungs. Since the host antigen material could not be separated from the virus particles by a variety of methods, it was concluded that influenza viruses contain such antigens as integral parts of

the virus structure. A similar conclusion was reached by Smith and colleagues as a result of extensive complement fixation tests (1955). Munk and Schäfer found an almost exactly parallel situation for fowl plague and Newcastle disease viruses grown in the chick embryo (1951). Thus, an incorporated host antigen seems to be characteristic of several viruses, especially those whose particles have an envelope structure.

With respect to the quantitative side of immunochemical tests, it should be noted that the quantitative precipitin test will give a figure for the amount of host antigen in the virus preparation (Kabat 1943). When the host antigen is incorporated in or tightly bound to the virus particles, this value may be only approximate, owing to changes in the number and reactivity of antibody-binding sites. Thus, it was calculated that influenza virus particles contain 20–30 percent host antigen (Knight 1946a), and that Newcastle disease virus contains about 42 percent (Munk and Schäfer 1951). If the host antigen and virus particles are not combined, it is possible, of course, to obtain a more precise estimate of the quantity of host antigen present. Furthermore, many antigens are detectable and can be quantitatively measured at 1 percent or less.

The popular gel diffusion serological method has found use in identifying and evaluating the purity of viruses (see, for example, LeBouvier et al. 1957; van Regenmortel 1966).

Finally, the importance of the immunochemical test as a criterion of purity is its independence of the factors basic to other tests, such as size, shape, and electrochemical properties. On the other hand, a limitation of the method that must be remembered is that not all potential impurities are good antigens; furthermore, the antihost constituent serum may not be sufficiently comprehensive to detect all possible contaminants. Nevertheless, the immunochemical method is one of the most powerful and sensitive means for evaluating the purity of a virus preparation.

One of the most exacting tests of homogeneity of proteins is the constant solubility test (Taylor 1953). However, this test is not used widely as a criterion of the purity of viruses because of restrictions imposed by the limited quantity of material available in many cases and technical difficulties in others. From the results of early solubility studies made on purified preparations of tobacco mosaic virus, Loring (1940) concluded that the virus is not a homogeneous chemical substance. Since that time, numerous refined chemical and structural studies made on this virus have demonstrated a surprising uniformity of its properties (Knight 1954; Tsugita et al. 1960), and lead one to conclude that there must have been something anomalous in the solubility studies or the preparations of virus used in these studies. Further work needs to be done along these lines.

In summary, no single criterion of purity is sufficient to establish the homogeneity of a preparation of virus. This must be done by applying critically as many tests as possible (see Knight 1974).

In all of the homogeneity procedures described, it is, of course, essential to try to relate the observed physical particles to the biological activity (infectivity) and to show that the characteristic particles are the biologically active ones. Many such tests of this character were made in establishing the identity of the infectious entity and the characteristic rodlike particles of tobacco mosaic virus (Stanley 1939). Lauffer and colleagues have described rigorous methods for relating biological activity to the physical particles observed in a variety of ways (Lauffer 1952; Epstein and Lauffer 1952; Hartman and Lauffer 1953; Shainoff and Lauffer 1957). Finally, with certain of the T-bacterial viruses it has been possible to make a good correlation between the particles counted with the electron microscope and the number of infectious units found by infectivity measurements (Luria et al. 1951).

III

Composition of Viruses

Several hundred virus diseases are now recognized as such, but the chemical compositions of relatively few viruses have been reported. The main reasons for this situation are that many interesting experiments can be done without precise information about viral composition, and it is not easy to obtain all viruses in a state of purity adequate for analysis. Finally, it is somewhat tedious to perform complete and thorough analyses of viruses even when they can be obtained in adequate amounts and in sufficient purity. Consequently, it is common for investigators to make just those analyses most pertinent to a particular topic under investigation.

Tobacco mosaic virus proved to be a nucleoprotein, that is, a specific combination of nucleic acid and protein. The same characteristic nucleoprotein was obtained from a variety of hosts infected with tobacco mosaic virus (TMV). In each instance the virus nucleoprotein appears to be foreign to its host as judged from its absence in normal plants and its lack of serological relationship to the normal host constituents. This holds true for many other viruses, although among the more complex viruses, those that acquire envelope structures at and bud out through cell membranes typically contain some host cell constituents in their envelopes.

Most viruses give rise during multiplication to occasional mutants, or variants; when isolated, these are found to be of the same general composition as that of the parent virus. It now seems generally true that strains of a given virus have identical proportions of protein and nucleic acid, although exceptions to this rule have been observed in mutants of the more complex viruses.

All types of viruses, including plant, bacterial, higher animal, and insect viruses, contain either ribonucleic acid (RNA) or deoxyribonucleic acid (DNA) (see Table 2). At one time it was thought that some viruses, such as influenza virus, contained both types of nucleic acid, but, as a consequence of more refined methods for purification and analysis of viruses, no virus is presently known that contains both RNA and DNA. This may be contrasted with bacteria including *Rickettsia* and *Bedsonia*, which appear to have both DNA and RNA (see Allison and Burke 1962). Some other comparisons between viruses and bacteria are given in Table 3.

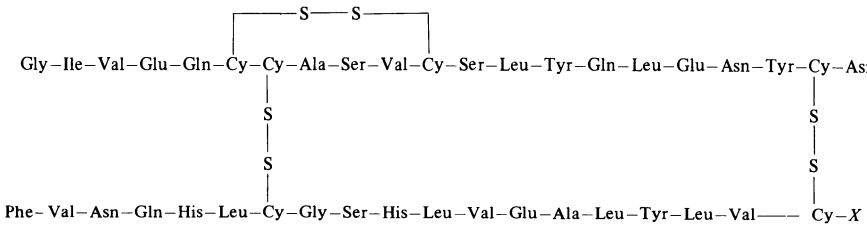
Almost all of the many plant viruses that have been obtained in a highly

purified state, as well as some small animal and bacterial viruses, have proved to be simple nucleoproteins. However, some of the larger animal and bacterial viruses also possess lipid, polysaccharide, and other components. It should be noted that a few plant and bacterial viruses have also been found to contain some lipid. The general compositions of some viruses are listed in Table 2.

A. Proteins

The structures of proteins, including those of viruses, are usually complex and hence require a series of studies to characterize them. A division of protein structure into four distinctive types can be made (see Kendrew 1959), each of which can be investigated experimentally:

1. *Primary structure*: the number of different peptide chains (determined mainly by end group analyses), interchain binding, if any (such as by S-S-bonds), and, most especially, the sequence of amino acid residues. These features of primary structure are illustrated by the formula for bovine insulin as determined by Sanger and associates (Ryle et al. 1955):



In order to save space, the last portion of the second peptide chain has been represented by *X*, in which *X* stands for -Gly-Glu-Arg-Gly-Phe-Phe-Tyr-Thr-Pro-Lys-Ala.

It can be seen from the formula that insulin has two peptide chains that are joined at two positions by disulfide bonds between cysteine residues, and the sequence of amino acid residues is as indicated. In this formula and in subsequent tables, the abbreviations commonly employed by protein chemists are used. A list of such abbreviations is given in Table 4.

2. *Secondary structure*: the geometric configuration of the peptide chain (or chains) with special reference to the presence or absence of helical structure. The commonest configuration of peptide chains is probably the so-called alpha helix of Pauling and associates (1951), which is illustrated in Figure 5. It will be noted that a spiral structure is favored by the formation of hydrogen bonds between adjacent -CO and -NH- groups.

3. *Tertiary structure*: the folding pattern of the peptide chain. Possibilities of folding (often the folding of a helical chain) are illustrated by

Table 2. Approximate Composition of Some Viruses.

Virus	% RNA	% DNA	Protein[a]	% Lipid	% Non-nucleic Acid Carbohydrate	Ref.[b]
Adenovirus		13	87			1
Alfalfa mosaic	19		81			2
Avian myeloblastosis	2		62	35	1[c]	3, 4
Blue-green algal LPP-1		48	52			5
Broad bean mottle	22		78			6
Brome mosaic	21		79			7
Carnation latent	6		94			8
Cauliflower mosaic		16	84			8
Coliphages f1, fd, M13		12	88			9
Coliphages f2, fr, M12, MS2, R17, QB, ZR	30		70			10
Coliphage ØX174, ØR, S13		26	74			9
Coliphages T2, T4, T6		55	40		5[d]	11, 12
Cowpea mosaic	33		67			8
Cucumber 3 (and 4)	5		95			13
Cucumber mosaic	18		82			8
Dasychira pudibunda L.	7		93			14
Encephalomyocarditis	30		70			15
Equine encephalomyelitis	4		42	54		16
Fowl plague	2		68	25	+	17
Herpes simplex		9	67	22	2	18, 18a
Influenza	1		74	19	6	19, 20
Mouse encephalitis (ME)	31		69			21
Pea enation mosaic	29		71			8
Poliomyelitis	26		74			22
Polyoma		16	84			23
Potato spindle tuber	100					24

Potato X	6		94			25
Reovirus	21		79		+	18
Rous sarcoma	2		62	35	1[c]	3, 4
Shope papilloma		18	82			23
Silkworm cytoplasmic polyhedrosis	23		77			26
Silkworm jaundice		8	77[e]			27
Simian virus 5	1		73	20	6	28
Southern bean mosaic	21		79			29
Tipula iridescent		13	82	5		30
Tobacco mosaic	5		95			31
Tobacco necrosis	19		81			32
Tobacco rattle	5		95			33
Tobacco ringspot	40		60			34
Tomato bushy stunt	17		83			35
Tomato spotted wilt	5		71	19	5	36
Turnip yellow mosaic	34		63			37
Vaccinia		5	88	4	3	38
Wild cucumber mosaic	35		65			39
Wound tumor	23		77			26

[a]Rounded figures, often obtained by difference between 100 percent and sum of other components.

[b](1) Green 1969; (2) Frisch-Niggemeyer and Steere 1961; (3) Bonar and Beard 1959; (4) Baluda and Nayak 1969; (5) Brown 1972; (6) Yamazaki et al. 1961; (7) Bockstahler and Kaesberg 1961; (8) Harrison et al. 1971; (9) Ray 1968; (10) Kaesberg 1967; (11) Kozloff 1968; (12) Thomas and MacHattie 1967; (13) Knight and Stanley 1941, Knight and Woody 1958; (14) Krieg 1956; (15) Faulkner et al. 1961; (16) Beard 1948; (17) Schäfer 1959; (18) Joklik and Smith 1972; (18a) Russell et al. 1963; (19) Ada and Perry 1954; (20) Frommhagen et al. 1959; (21) Rueckert and Schäfer 1965; (22) Schaffer and Schwerdt 1959; (23) Kass 1970; (24) Diener 1971; (25) Bawden and Pirie 1938; (26) Kalmakoff et al. 1969; (27) Bergold and Wellington 1954; (28) Klenk and Chopin 1969a; (29) Miller and Price 1946; (30) Thomas 1961; (31) Knight and Woody 1958; (32) Kassanis 1970; (33) Harrison and Nixon 1959a; (34) Stace-Smith 1970; (35) Stanley 1940, DeFremery and Knight 1955; (36) Best 1968; (37) Matthews 1970; (38) Fenner et al 1974; (39) Yamazaki and Kaesberg 1961.

[c]A rough estimate calculated from data of Baluda and Nyak (1969).

[d]Hydroxymethyl cytosine residues of the T-even DNAs are glucosylated but to different extents so that the glucose residues amount to about 4, 5, and 7 percent, respectively, of the DNAs of T2, T4, and T6 (Jesaitis 1956). An average value for DNA and carbohydrate is given here.

[e]About 15 percent of the virus was not accounted for as protein, nucleic acid, or lipid.

Table 3. Some Comparisons of Viruses and Bacteria.[a]

Microorganism	Size, nm (Approx. Diam.)	Chemical Composition	Multiplication	Inhibition by Antibiotics	Staining Characteristics
Bacteria	500–3,000	Complex: numerous proteins (including enzymes), carbohydrates, fats, etc.; DNA and RNA; cell wall contains mucopeptide	In fluids, artificial media, cell surfaces, or intracellularly, by binary fission	Inhibited	Stain with various dyes
Mycoplasmas or PPLOs[b]	150–1,000	Similar to other bacteria but generally possess no cell wall	In media similar to other bacteria but by budding rather than fission	Resistant to penicillins, sulfonamides; sensitive to tetracyclines, kanamycin, etc.	Stain with dyes but poorly
Rickettsia	250–400	Similar to other bacteria	Inside living cells by binary fission; major hosts: arthropods	Inhibited	Stain with various dyes
Chlamydia or *Bedsonia*	250–400	Similar to other bacteria	Inside living cells by binary fission; major hosts: birds and mammals	Inhibited	Stain with various dyes
Viruses	15–250	Mainly nucleic acid (one type) and protein. Some contain lipid and/or carbohydrate in addition	Inside living cells by synthesis from pools of constituent chemicals	Not inhibited	Stain for electron microscopy with salts of heavy metals

[a]Adapted from Knight 1974.
[b]PPLO is the abbreviation for pleuropneumonia-like organism, the first of this group of wall-less bacteria to be characterized.

Table 4. Names and Abbreviations of Common Amino Acids.

Alanine	Ala	Leucine	Leu
Arginine	Arg	Lysine	Lys
Asparagine	Asn	Methionine	Met
Aspartic acid	Asp	Phenylalanine	Phe
Cysteine	Cys	Proline	Pro
Glutamic acid	Glu	Serine	Ser
Glutamine	Gln	Threonine	Thr
Glycine	Gly	Tryptophan	Trp
Histidine	His	Tyrosine	Tyr
Isoleucine	Ile	Valine	Val

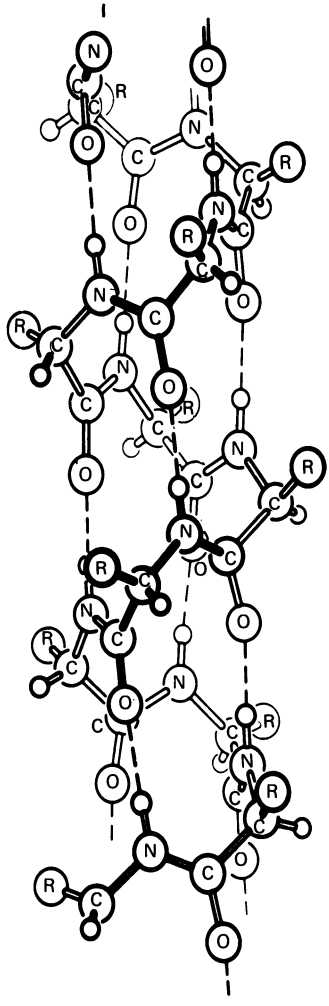


Fig. 5. The α-helix of Pauling et al. (1951). Note the formation of hydrogen bonds, indicated by dashed lines, between -CO and -NH groups. There are 3.7 amino acid residues per complete turn, and a unit residue translation of 1.47 Å giving a pitch of 5.44 Å. (From Kendrew 1959.)

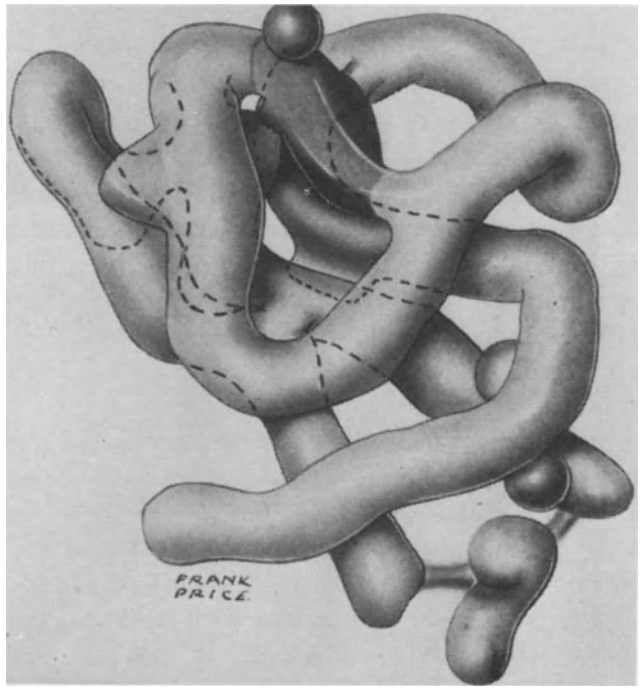


Fig. 6. Model of the myoglobin molecule derived from the three-dimensional electron density map based on x-ray data. The polypeptide chain is represented by solid rods; the side chains have been omitted but if present would fill the regions between the main chains. The small spheres are the heavy atoms used to determine the phases of the x-ray reflections. (From Kendrew 1959.)

the tertiary structure of myoglobin determined from crystallographic studies and represented by the model shown in Figure 6 (Kendrew 1959).

4. *Quaternary structure*: the number and spatial relationship of repeating subunits when these are present. An excellent example of quaternary structure is the architecture of the tobacco mosaic virus particle (see the model in Figure 7).

Primary structure is the stablest of the four types since this kind of structure involves covalent bonds, whereas the others depend on secondary attractions, that is, ionic and hydrophobic interactions and hydrogen bonds. The so-called "denaturation" of proteins results from disruption of secondary bonds and is manifested by the unfolding of the protein and changes in the solubility, charge, hydration, and serological and chemical reactivities. Rupture of secondary bonds affects quaternary as well as tertiary structure, and is the means for disaggregating virus particles, as will be described next.

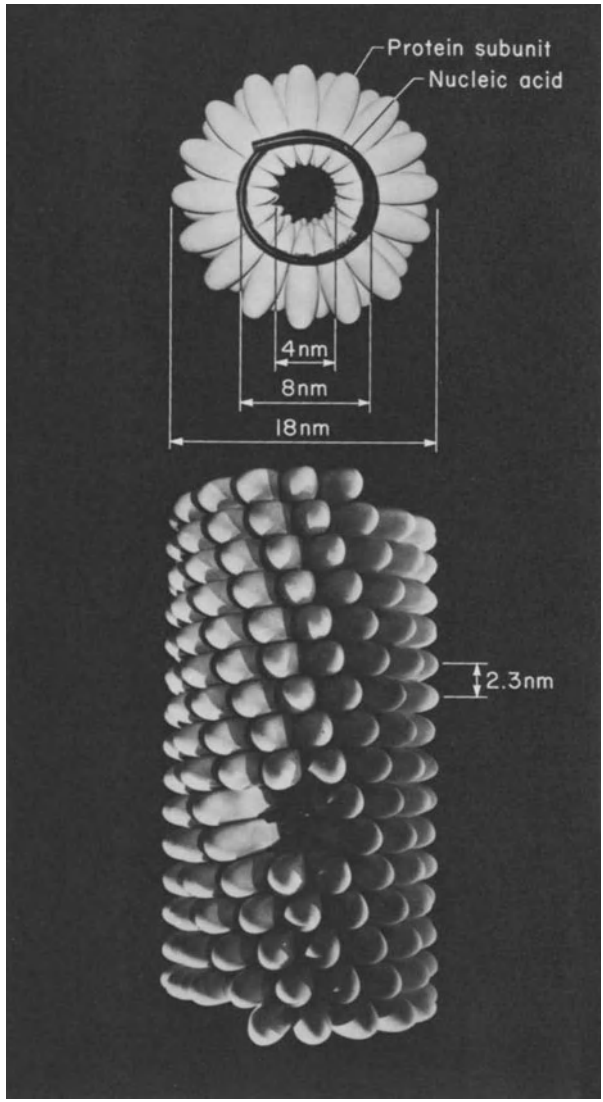


Fig. 7. Model of about one-tenth of the tobacco mosaic virus particle. The protein subunits are schematically illustrated in a helical array about the long axis of the particle. The structure repeats after 6.9 nm in the axial direction, and the repeat contains 49 subunits distributed over three turns of the helix of 2.3 nm pitch. Some of the subunits have been removed in order to show the deeply embedded location of the viral RNA, which, however, is better viewed in cross section as shown at the top of the illustration.

1. *Preparation of Viral Proteins*

A first step in determining primary structure is to obtain an accurate analysis of the amino acid content of the protein. This may be done with hydrolysates of the whole virus, but more reliable results are generally obtainable if the viral protein is first separated from the other viral constituents. This removes nonprotein materials whose acid-degraded products may interfere with the analytical methods, and eliminates spurious glycine that can arise from acid degradation of the nucleic acid (see Smith and Markham 1950). Isolation of proteins before analysis is mandatory for the many viruses that contain more than one species of protein.

Each virus presents a separate problem with respect to appropriate means for disrupting quaternary structure (without breaking covalent bonds) and subsequent isolation of homogeneous protein preparations. Obviously, the most complicated situations arise with such viruses as the myxoviruses (influenza, Newcastle disease, mumps) and bacterial viruses, which possess not one but several discrete protein components, as well as a variety of other chemical constituents. However, it has been found with several viruses that disruption of quaternary structure and resultant release of protein occur upon treatment of virus with acid, alkali, or/and detergent. Also, treatment with salts or with such reagents as urea, phenol, or guanidine hydrochloride have occasionally been used with success. Some of these methods will be summarized.

a. The Mild Alkali Method (Schramm et al. 1955; Fraenkel-Conrat and Williams 1955)

Exposure of TMV in the cold to dilute alkali at pH 10–10.5 results in release of protein, which, from its sedimentation and diffusion characteristics, appears to have a molecular weight of 90,000–100,000 (that is, it is either a pentamer or hexamer of the fundamental protein subunit). This material has been called "A protein" (Schramm et al. 1955). At the same time, the RNA of the virus undergoes alkaline hydrolysis to small fragments.

The quality of a good preparation of A protein is apparently so similar to that of the native protein in the virus particle that it can be used, together with viral RNA, to reconstitute (see section on Reconstitution of Viruses) virus rods that are virtually indistinguishable from undegraded virus.

The alkali employed appears not to be critical since sodium hydroxide, borate, carbonate, and glycine buffers have all been used with good results. The amino alcohols, such as ethanolamine, seem to be more effective than alkaline buffers in degrading the virus and hence achieve the desired result in a shorter time (Newmark and Myers 1957).

A convenient alkaline degradation procedure used successfully with TMV and many of its strains is as follows:

An aqueous solution of virus at 10 mg/ml is placed in a cellophane bag and dialyzed at about 4° against 2 liters of 0.1 M carbonate buffer (21.2 g Na_2CO_3 in 2 liters H_2O adjusted to pH 10.5 by addition of solid $NaHCO_3$) for 2–5 days. Alternatively, 0.02 M ethanolamine at the same pH and temperature can be used with a reduction of the dialysis time to 2–4 hr.

Separate undegraded virus by centrifugation of the contents of the dialysis bag at 60,000–100,000 *g* for 1 hr. Discard the pellet and add 1 vol of saturated ammonium sulfate to the supernatant fluid. Sediment the precipitated protein at about 5,000 *g* and redissolve in water. Precipitate twice more with 0.33 saturated ammonium sulfate and dialyze at 4° against several changes of distilled water. Adjust the pH of the dialyzed solution to 7–8 with dilute NaOH and centrifuge at 60,000–100,000 *g* for an hour to remove heavy particles. Store the final solution in the refrigerator, adding a drop or two of chloroform as preservative. The ultraviolet absorption ratio of the maximum to the minimum (280/250) should be about 2 or higher, depending mainly on the tryptophan and tyrosine contents of the protein.

The kinetics of the degradation of the quaternary structure of TMV in mildly alkaline solutions have been studied with the use of the ultracentrifuge and viscometry (Schramm 1947a; Schramm et al. 1955; Harrington and Schachman 1956). Some conclusions of general interest that Harrington and Schachman reached are:

1. The character of the alkaline degradation of TMV changes markedly between 0° and 25°, and different products are obtained at the two temperatures.
2. Contrary to earlier interpretations, some of the intermediates and some of the final products are the result not of degradation of larger components but rather of aggregation of smaller degradation products.
3. TMV seems to possess structural features of such a nature that the protein subunits are rapidly stripped at pH 9.8 from two-thirds of the particle leaving a relatively stable nucleoprotein fragment one-third the initial size of the particle.
4. Some particles of TMV seem to be completely resistant to degradation under conditions that lead to the breakdown of the bulk of the virus.

Conclusion 4 had also been reached earlier by Schramm et al. (1955), and remains a puzzle, along with the seemingly greater stability to alkali of one-third of the particle. Possibly the interaction between protein and nucleic acid is not uniform along the length of the rod, being stronger at one end than at the other.

b. The Cold 67 Percent Acetic Acid Method (Bawden and Pirie 1937; Fraenkel-Conrat 1957)

The proteins of some strains of TMV are too sensitive to alkali to permit their isolation from the virus at high pH values. In such cases the prepara-

tion of viral protein may be facilitated by use of the acid degradation procedure developed by Fraenkel-Conrat (1957), and described as follows:

To ice-cold virus solution of 10–30 mg/ml in water is added 2 vol of chilled glacial acetic acid, and the mixture is allowed to stand in an ice bath for 30–60 min, with occasional stirring. The nucleic acid separates out while protein stays in solution. The nucleic acid is removed by centrifuging in the cold and the protein is then dialyzed against several changes of distilled water at 4° for 2–3 days, by which time the protein may have reached its isoelectric range and have come out of solution. If the protein remains soluble, a series of precipitations with ammonium sulfate should be employed for completion of the preparation as described in the mild alkali method above. Otherwise, the isoelectrically precipitated protein is removed from the dialysis bag and pelleted by centrifugation; the pellet is dissolved in distilled water by adjusting to pH 8 with dilute NaOH and the solution is centrifuged at about 100,000 *g* for an hour to remove any undegraded virus or denatured protein. The water-clear supernatant, which contains the protein, may be used as such or dried from the frozen state and stored for use.

The acetic acid method did not work satisfactorily with turnip yellow mosaic virus (Harris and Hindley 1961) because insoluble protein aggregates were obtained. These aggregates were apparently formed by oxidation of SH groups to give S-S linkages between protein subunits. However, by converting the SH groups to carboxymethyl-SH with iodoacetic acid, subsequent aggregation was avoided, and the procedure could be carried out successfully as described above.

A useful modification of the acetic acid method is to substitute formic acid at 37° and to treat for 18 hr (Miki and Knight 1965).

c. The Guanidine Hydrochloride Method (Reichmann 1960; Miki and Knight 1968)

Some viruses are not readily dissociated by treatment with acid or alkali but do respond to protein-denaturing agents such as guanidine hydrochloride and urea. The former has proved more useful with both plant and animal viruses.

Dialyze virus at 10–20 mg/ml against 2 M guanidine HCl at room temperature overnight. During dialysis, the nucleic acid precipitates and the protein remains in solution. The nucleic acid is removed by centrifugation at 5,000 *g* and the supernatant is dialyzed against water for one or two days. The dialyzed material is centrifuged at 100,000 *g* for 2 hr in order to remove incompletely degraded virus. The yield of protein from potato virus X is about 90 percent.

A somewhat modified method is applied to poliovirus (Scharff et al. 1964) consisting of treatment with 6.5 M guanidine HCl at pH 8.3 for 3 hr at

37° followed by separation of protein and nucleic acid by centrifugation on a sucrose density gradient.

d. The Warm Salt Method (Kelley and Kaesberg 1962)

This procedure has been used successfully in preparing protein subunits from alfalfa mosaic virus, a small rodlike plant virus containing about 81 percent protein and 19 percent RNA.

Combine the virus at about 20 mg/ml in 0.01 M phosphate buffer at pH 7 with an equal volume of 2 M NaCl; hold the resultant mixture at 45° for 20 min. (During this time the solution becomes turbid, presumably because the released protein is less soluble than whole virus in M NaCl.) Cool immediately and centrifuge at low speed. The protein is sedimented while the nucleic acid and/or its degradation products remain in the supernatant fluid. Wash the protein pellet with M NaCl and centrifuge again. Dissolve the protein pellet in 0.01 M phosphate at pH 7 containing 0.005 M sodium dodecyl sulfate. Dialyze for 24 hr against distilled water and then precipitate by adding 0.66 vol of saturated ammonium sulfate. Resuspend the precipitate in phosphate buffer containing 0.05 M dodecyl sulfate, and then dialyze for 24 hr against phosphate buffer containing 0.005 M sodium dodecyl sulfate.

The molecular weight of the protein obtained by this method from alfalfa mosaic virus was estimated from sedimentation data to be about 34,000 (Kelley and Kaesberg 1962). The preparation was also found to be serologically active when tested with antiserum to whole alfalfa mosaic virus. This would not have been the case had the tertiary structure been extensively disrupted.

e. The Cold Salt Method (Yamazaki and Kaesberg 1963)

This procedure has been applied successfully to bromegrass mosaic and broad bean mottle viruses with recoveries of 60–80 percent of the viral protein.

The virus, at about 10–15 mg/ml in water, is dialyzed against 1 M $CaCl_2$ at pH 6–7 at 4° for 12 hr. During dialysis a white precipitate of nucleate is formed. The suspension is centrifuged at 5,000 *g* for 20 min. The supernatant is dialyzed against water to remove $CaCl_2$. The dialyzed material contains the soluble protein but practically no nucleic acid.

f. The Phenol Method (Anderer 1959a, 1959b)

Disruption of viruses with phenol is the basis for one of the commonest methods for isolation of viral nucleic acids (see section on Methods for Preparing Viral Nucleic Acids). In the preparation of nucleic acids the protein and other phenol-soluble components are usually discarded in the phenolic layer. However, it has been shown with TMV that protein can be

recovered from the phenolic layer, and, by suitable treatment, be restored to a condition which resembles the native state (Anderer 1959b).

To the phenolic layer remaining after separation of the aqueous, RNA-containing layer (see section on The Phenol Method for Preparing Nucleic Acid), add 5–10 vol of methanol and a couple of small crystals of sodium acetate. Remove the precipitate by centrifugation and wash three times with methanol and once with ether. Dry the product in air. To solubilize the air-dried protein, suspend 10 mg in 5 ml of water and heat at 60°–80°, adding enough 0.02 N NaOH to bring the pH to 7.5. The protein should dissolve and remain in solution upon cooling.

The TMV protein is denatured after extraction with phenol, precipitation with methanol, and so on, as described above. However, renaturation is assumed to occur to a large extent when the protein is warmed at about 60° at pH 7–7.5.

g. The Detergent Method and General Conclusions

It was early noted (Sreenivasaya and Pirie 1938) that 1 percent sodium dodecyl sulfate (SDS) disrupts TMV over a wide pH range. Later, when disruption by SDS was coupled with fractional precipitation with ammonium sulfate, Fraenkel-Conrat and Singer (1954) showed that the protein and nucleic acid of TMV could be rather cleanly separated. However, there are at least two disadvantages in the protein prepared by treatment of virus with SDS:

1. The protein strongly binds as much as 15 percent SDS, which seems to introduce only relatively small errors in ultracentrifuge studies (see Hersh and Schachman 1958, for example), but is more serious for other types of investigation. For example, trypsin is inhibited by anionic detergents such as SDS (Viswanatha et al. 1955), and TMV protein prepared by treatment with SDS does not appear to be satisfactory for structural studies dependent on a quantitative cleavage of protein by trypsin (Fraenkel-Conrat and Ramachandran 1959).

2. In addition to effecting the release of protein subunits, SDS tends to degrade the secondary and tertiary structures in a not readily reversed manner. Thus, TMV protein prepared with SDS is insoluble from pH 2 to 10 and does not participate in reconstitution (see section on Reconstitution of Viruses). However, the use of detergents, alone or in combination with other reagents such as phenol, has been invaluable in the disruption of virus particles for the isolation of viral nucleic acids (see Preparation of Nucleic Acids).

In general, all of the methods used to prepare viral proteins probably cause various denaturative changes, some of these reversible and others not. In the case of TMV, Anderer (1959b) has suggested the following criteria for distinguishing between native and denatured protein. Native TMV protein (1) is soluble in neutral aqueous media, (2) aggregates to

viruslike rods at pH 5–7, (3) reconstitutes to infectious virus with appropriate viral RNA, and (4) resembles the protein in the virus in amount of TMV antibody it binds. These criteria apply more or less to all viruses.

2. *Analysis of Viral Proteins*

The structure of proteins, as mentioned earlier, can be considered to fall into four main categories, each with its methods of analysis. Main consideration will be given here to the determination of primary structure because this is basic to the other types of structure. Thus the degree of helicity exhibited by the polypeptide chain (secondary structure), the nature of its folding (tertiary structure), and the assembly of protein subunits to form superstructures of characteristic morphology (quaternary structure) are virtually predestined by the sequence of amino acids in the protein chains. Some details of quaternary structure are considered in Sec. IV, Morphology of Viruses.

a. Amino Acid Analyses

In order to determine the composition of a viral protein, it is necessary, as with other proteins, to release the constituent amino acids by hydrolysis. This hydrolysis is usually accomplished by heating under vacuum 2–5 mg of protein in 1 ml of 6 N HCl in a thick-walled, sealed glass tube at 110° for 22–72 hr (see Knight 1964) (some proteins can be satisfactorily hydrolyzed at 120° for 6–24 hr; see Carpenter and Chramback 1962). Tryptophan and cysteine are largely destroyed by these conditions but can be preserved by modifying the hydrolysis medium (see Liu 1972; Liu and Inglis 1972). Alternatively, there are colorimetric procedures for determining tryptophan and cysteine (Anson 1942; Spies and Chambers 1949).

Customarily, two or more different times of hydrolysis are employed for evaluation of the release and recovery of individual amino acids. The highest value observed in a series or by extrapolation of observed values is generally accepted. Thus, as hydrolysis time increases, serine and threonine, and sometimes tyrosine, tend to be proportionately more destroyed; therefore, the contents of these amino acids are usually calculated by extrapolation back to zero time from the values observed at different times of hydrolysis. Conversely, when two or more residues of isoleucine or valine—and, to some extent, leucine—are contiguous, they are less readily released from peptide linkage than other amino acids. Thus even maximum periods of hydrolysis may yield somewhat low values. When the nature of the results suggests this, extrapolation to higher values is generally done by inspection and approximation. The uncertainties connected with such approximations are virtually eliminated if analyses can be made of the tryptic peptides of a protein (see Table 5) because the numbers of residues are

Table 5. Amino Acid Content of Tryptic Peptides of Tobacco Mosaic Virus.

Amino Acid	Tryptic Peptide[a] 1	2	3	4	5	6	7	8	9	10	11	12	Res.[b] Sum	Residue M. W. Sum
Ala	4	0	0	0	0	3	0	3	2	1	0	1	14	995.05
Arg	1	1	1	0	1	1	1	1	2	1	1	0	11	1,718.15
Asn	3	0	0	0	0	1	1	2	0	2	1	0	10	1,141.05
Asp	1	0	0	2	0	2	0	1	2	0	0	0	8	920.68
Cys	1	0	0	0	0	0	0	0	0	0	0	0	1	103.12
Gln	5	1	3	0	0	0	0	1	0	0	0	0	10	1,281.35
Glu	1	0	0	0	0	0	0	3	0	1	0	1	6	774.69
Gly	1	0	0	0	0	1	0	0	0	0	2	2	6	342.33
Ile	3	0	0	0	0	0	0	2	1	3	0	0	9	1,018.49
Leu	4	0	0	0	0	4	0	1	0	2	0	1	12	1,357.98
Lys	0	0	1	1	0	0	0	0	0	0	0	0	2	256.35
Phe	3	0	1	2	0	1	0	0	0	0	1	0	8	1,177.40
Pro	2	0	2	1	0	1	0	1	0	0	0	1	8	776.92
Ser	5	0	2	1	0	0	0	0	0	1	1	6	16	1,391.60
Thr	4	1	1	0	0	2	0	4	1	0	1	2	16	1,617.68
Trp	1	0	1	0	0	0	0	0	0	0	0	1	3	558.62
Tyr	1	0	0	0	1	1	0	0	0	0	1	0	4	652.70
Val	1	2	3	0	1	2	0	1	2	1	0	1	14	1,387.89
Totals													158	17,472.05[c]

[a]The 12 peptides resulting from treatment of tobacco mosaic virus coat protein with trypsin are numbered in order from the N-terminal to the C-terminal of the polypeptide chain.

[b]Res. = Residue. An amino acid residue is the molecular weight of the amino acid less one molecule of water.

[c]The N-terminal amino acid of TMV protein is acetylated. If the molecular weight of the acetyl group and a hydroxyl associated with the C-terminal threonine are added on to the sum of the residues, a value of 17,516.08 is obtained. For most practical purposes, a rounded value of 17,500 can be used for the TMV coat protein.

fewer and systematic errors are less significant. For example, if a peptide has 4 Ala residues, a 3 percent error in its analysis amounts to a negligible ± 0.1 Ala residue, whereas if the whole protein contains 20 Ala residues the error is ± 0.6 residue, which entails an uncertainty of ± 1 residue from the apparent value.

The amino acids resulting from hydrolysis of proteins are generally determined quantitatively in commercial, automatic amino acid analyzers such as that in Figure 8. These machines are an outgrowth of the laboratory models first developed and used effectively in amino acid analyses by S. Moore and W. H. Stein and associates at the Rockefeller University in New York (see Spackman et al. 1958; Spackman 1967).

In operation, something between a few microliters and a milliter or more of hydrolysate is applied at the top of a column of polysulfonic cation exchange resin and then appropriate buffers are pumped through resin

under high pressure. In passage through the resin, the various amino acids are repeatedly adsorbed and eluted at rates dependent upon their chemical composition. As the amino acid fractions emerge from the column, they undergo reaction with a ninhydrin solution and the resulting color is measured by passage through a colorimeter whose readings are recorded automatically on a chart such as that shown on the instrument in Figure 8.

The various amino acids are identified by the order in which they emerge from the column as indicated by the successive peaks observed on the recorder chart, and the quantity of each amino acid is obtained by comparison of the areas under the various peaks with the areas obtained with known quantities (for example, 0.1 μM) of standard amino acids. This comparison is made through a series of calculations as specified by the manufacturer of the analyzer, but is facilitated in some instruments by an integrator accessory that automatically integrates the areas under the respective peaks; there is also a system which translates recorder output to a

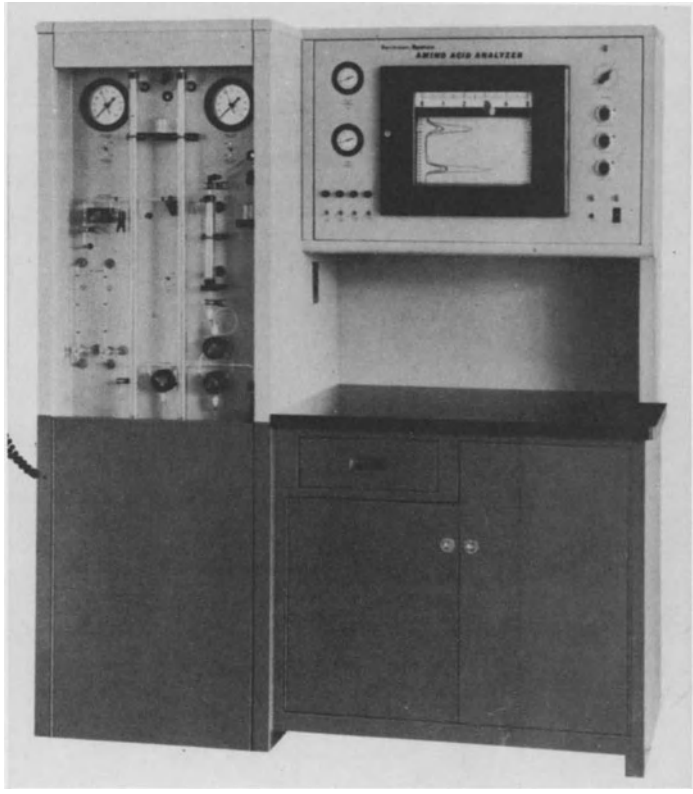


Fig. 8. Beckman amino acid analyzer. Note the cylindrical glass columns for the ion exchange resin on the left and the chart recorder on the right.

form suitable for subsequent processing in a digital computer (see Hirs 1967). Figure 9 illustrates the peaks obtained in a run made with a standard mixture containing 0.1 μM of each of the commonest amino acids.

The results from analyses made with amino acid analyzers are in terms of micromoles of each amino acid present in the applied sample. For maximum usefulness in structural and genetic analyses, these values are converted to numbers of each amino acid residue present per protein molecule (an amino acid residue is an amino acid minus the elements of water that are lost in the incorporation of the amino acid into a polypeptide chain). This conversion is done as follows.

First, a minimal molecular weight is calculated for the protein. In the absence of any estimate of the molecular size of the protein, this calculation involves a series of tentative assignments whose purpose is to find the lowest level at which the various amino acid residues appear in integral numbers. To start, a value of 1 can be assigned to the amino acid present in lowest amount (or the value of 1 can be assigned to any amino acid characterized by a consistently high recovery from protein hydrolysates) and the number of each of the other amino acid residues can be calculated on the basis of their relative micromolar values. Thus, by trial, a set of residue

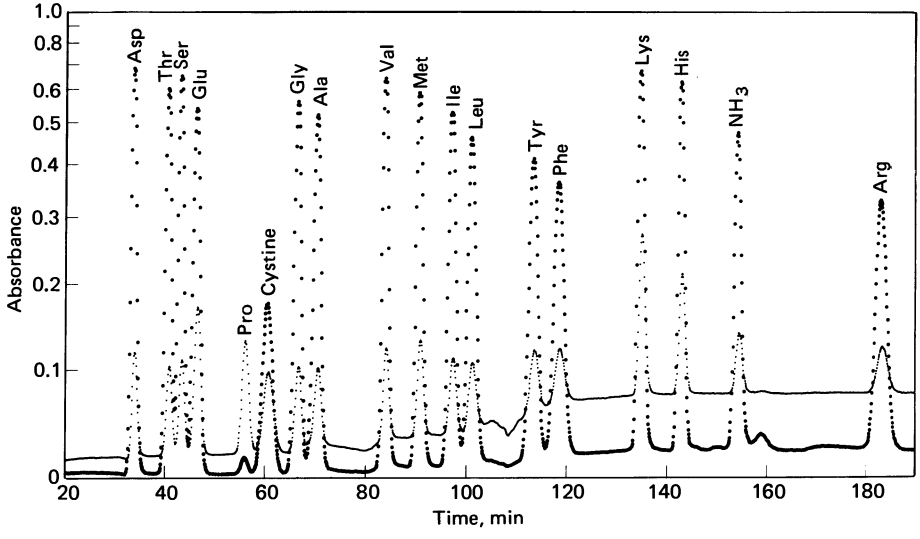


Fig. 9. Tracing from a Beckman amino acid analyzer chart showing the curves obtained with approximately 1 μM of each amino acid. The heavy dots represent the absorbance values at 570 nm and the small dots at 440 nm of the colors obtained in the reaction of each amino acid with ninhydrin. This color is purple for all common amino acids except proline, which yields a yellow product. Note that it took only about 3 hr for a complete analysis of the mixture of amino acids represented here.

numbers can be obtained in which the values are nearly whole numbers for all of the amino acids present. Summation of these residue weights yields a minimal molecular weight for the protein.

At this point, an independent estimate of the molecular size is needed in order to determine the factor by which the minimal numbers of residues must be multiplied to give the actual numbers present per molecule of viral protein. Some procedures to estimate molecular weights of proteins accurately enough for this use include (1) end group analysis (see Sec. IIIA, 2b), acrylamide gel electrophoresis (see Sec IIIA, 2c), agarose gel chromatography (see Sec. IIIA, 2c), and tryptic digestion followed by peptide mapping and counting (see Sec. IIIA, 2c). The molecular weight obtained by one or more of these methods divided by the minimal molecular weight yields a figure whose nearest integer is the factor by which the numbers of residues must be multiplied to give actual numbers per protein molecule and by which the minimal molecular weight must be multiplied to give the actual molecular weight.

In current practice, some estimate of the molecular weight of the protein is usually made early in the process by one of the methods listed above (for example, gel electrophoresis) and this figure is used with results of the amino acid analyses to make the approximations leading to assignment of residue numbers. This type of analysis can be illustrated by data obtained with the protein of tobacco mosaic virus (Table 6).

Similar procedures have been applied to the coat proteins of numerous plant viruses (see Tsugita and Hirashima 1972) and to some bacterial virus coat proteins; analysis of animal virus proteins in terms of amino acid residues has received almost no attention. Some examples of the results obtained with plant and bacterial viruses are given in Tables 7 and 8.

If the data in Tables 7 and 8 are compared with those for nonviral proteins (see, for example, Dayhoff 1972), it appears that viral proteins have ordinary quantities of the common amino acids and that no unusual amino acids have yet been observed in them. Furthermore, the general agreement between results of amino acid analyses made many years ago by microbiological assay (which detects only the L isomers, that is, the form generally present in proteins of all sorts) and more recent results obtained by methods that do not distinguish between optical isomers suggests that the amino acids of viral proteins are primarily if not exclusively the L isomers.

It is also apparent from the values shown in Tables 7 and 8 that viral coat proteins, while containing significant quantities of the basic amino acids arginine and lysine, do not have sufficient quantities of these to place them in the protamine or histone class of proteins. The latter often have been found in sperm and similar nucleoproteins. Actually, the isoelectric points reported for viruses are consistent with the idea that at least some viral proteins are acidic rather than basic. Some examples are given in Table 9.

Table 6. Amino Acid Analysis of Tobacco Mosaic Virus Coat Protein.

Amino Acid	Micromoles Amino Acid Found after Hydrolysis in 6N HCl at 120°C for				Maximum Micromoles	Number of Residues on Basis of			Best Estimate[c]
	6 hr	24 hr	48 hr	96 hr		Lys=2	Gly=6	Glu=16	
Asp	0.1773	0.1764	0.1755	0.1754	0.1773	18.96	18.21	18.31	18
Thr	0.1470	0.1337	0.1120	0.0953	0.1520[a]	16.26	15.61	15.70	16
Ser	0.1430	0.1119	0.0716	0.0493	0.1540[a]	16.47	15.82	15.91	16
Glu	0.1545	0.1547	0.1547	0.1549	0.1549	16.57	15.91	16.00	16
Pro	0.0799	0.0775	0.0827	0.0799	0.0799[b]	8.55	8.21	8.25	8
Gly	0.0584	0.0584	0.0582	0.0579	0.0584	6.25	6.00	6.03	6
Ala	0.1365	0.1349	0.1360	0.1348	0.1365	14.60	14.02	14.10	14
Val	0.1310	0.1386	0.1386	0.1385	0.1386	14.81	14.23	14.31	14
Ile	0.0744	0.0806	0.0822	0.0812	0.0822	8.79	8.44	8.49	9
Leu	0.1150	0.1185	0.1193	0.1184	0.1193	12.76	12.26	12.32	12
Tyr	0.0378	0.0384	0.0379	0.0354	0.0384	4.11	3.94	3.97	4
Phe	0.0763	0.0783	0.0789	0.0755	0.0789	8.44	8.11	8.15	8
Lys	0.0182	0.0176	0.0187	0.0187	0.0187	2.00	1.92	1.93	2
Arg	0.1045	0.1014	0.1028	0.0981	0.1045	11.18	10.74	10.79	11

[a]Values for Thr and Ser are obtained by extrapolation back to zero time of the plot of micromoles versus time of hydrolysis.

[b]The 48-hr value was discarded as anomalous.

[c]The best estimate is obtained by a series of test calculations. Such calculations were begun here by inspecting the list of maximum micromoles (column 5) of amino acid shown by the analyses and setting the amino acid occurring in smallest amount (Lys) equal to unity. When the residues of the other amino acids were calculated on the basis of Lys as 1, a value of 5.59 Arg was among those obtained. This gives a total of six or seven basic amino residues per protein subunit, and leads to the prediction that about this same number of peptides should result from tryptic digestion of the protein. In fact, nearly twice this number was observed. This suggests setting the Lys at 2, and the results of this assumption are shown in column 6. Inspection of these values shows that there are several that are far from integral and indicates that it might be more accurate to shift to an amino acid which is present in greater numbers of residues than Lys in order to reduce the effect of analytical error on the micromolar unit value. Glycine, which is known to be quantitatively released by and stable to acid hydrolysis conditions, was selected for the next test calculation (set at 6 from the first approximation) and the results yielded are shown in column 7. Values close to integral ones were obtained with almost all amino acids except isoleucine; when a shift was made to Glu as 16, the results were close to those obtained with Gly as 6 (column 8). Hence, the nearest integral values were assigned for each amino acid residue except isoleucine, which was assigned the next highest value on the basis of experience. The results are shown in the last column of the table. [It should be noted that neither histidine nor methionine is present in this protein but tryptophan and cysteine occur and as in proteins in general (see text) must be determined by independent analyses.]

b. Protein End Groups

A protein or peptide chain consists of a linear sequence of amino acid residues with two ends. In the usual manner of writing linear formulas for such structures, the amino acid residue on the extreme left is called the amino terminal, or N terminal, residue, whereas the amino acid residue on the extreme right is called the carboxyl terminal, or C terminal, residue. These terminal groups can be identified by cleaving them chemically or enzymatically from the protein chain followed by application of a method for amino acid analysis.

Knowledge about primary structure of viral proteins accumulated most rapidly with TMV, and the information obtained served as a model for the investigation of other viruses. For some years after Stanley's discovery of the nature of TMV, there was little interest in the primary structure of the virus protein, mainly because most investigators were overwhelmed with the idea of working with a protein whose apparent molecular weight was of the order of 38×10^6. However, the first step in primary structure work, namely, the determination of amino acid content of TMV and related viruses, was taken in Stanley's laboratory by Ross (Ross and Stanley 1939; Ross 1941) and was further developed in the same laboratory by Knight (Knight and Stanley 1941; Knight 1947b).

At the same time, evidence was gradually provided by physicochemical studies on the degradation products obtained from TMV with urea, alkali, and detergents (Stanley and Lauffer 1939; Lauffer and Stanley 1943; Wyckoff 1937; Schramm 1947a; Sreenivasaya and Pirie 1938) that the virus might possess a substructure. This idea was well supported by the classical x-ray diffraction studies that Bernal and Fankuchen (1941) made on TMV and other plant viruses. However, it was not clear from the results of the chemical degradation studies precisely what sorts of bonds were being broken, nor was it possible to define chemically the crystallographic subunits. Therefore the concept of viral protein subunits lay dormant until some years later.

A fresh approach to the question of viral subunits was launched with the attempt to determine the number and nature of the peptide chains in TMV by means of protein end group studies using the enzyme carboxypeptidase A.

Two pancreatic carboxypeptidases, A and B, carboxypeptidase C, found in citrus and a variety of other plants, and carboxypeptidase Y from yeast are known (see Ambler 1972; Hayashi et al. 1973). These differ in the rate at which they release specific terminal amino acids.

Carboxypeptidase A catalyzes the rapid release of terminal Ala, Gln, His, Ile, Leu, Met, Phe, Thr, Trp, Tyr, and Val; the slow release of Asn, Asp, Cys, Glu, Gly, Lys, and Ser; and generally fails to hydrolyze terminal Arg and Pro. In addition to their own refractory response to carboxypep-

Table 7. Amino Acid Residues Per Subunit of Some Plant Virus Coat Proteins.

Amino Acid	Virus									
	Brome Mosaic	Cowpea Chlorotic Mottle	Cucumber Mosaic	Cucumber 3 (Japan)	Cucumber 4	Potato X	Tobacco Mosaic	Tobacco Necrosis[d]	Tobacco Necrosis Satellite[e]	Turnip Yellow Mosaic
Ala	33	27	17	21	18	46	14	13	9	15
Arg	13	8	24	8	9	10	11	14	24	3
Asn							10			3
Asp	10[a]	11[a]	30	20[a]	17[a]	24[a]	8	18[a]	27[a]	7
Asx[b]										1
Cys	1	2	0	0	0	3	1	2	2	4
Gln							9			3
Glu	18[a]	17[a]	20[a]	10[a]	10[a]	19[a]	7	20	18	8
Glx[b]										3
Gly	10	10	16	9	5	13	6	8	8	8
His	4	2	4	1	0	2	0	1	6	3
Ile	8	7	16	7	5	12	9	11	13	15
Leu	15	16	26	18	13	10	12	10	20	17
Lys	13	13	18	4	3	12	2	12	11	7
Met	3	1	8	0	0	8	0	6	4	4

Phe	5	4	7	9	10	12	8	12	11	5
Pro	7	7	18	6	8	18	8	15	4	20
Ser	13	16	32	24	22	17	16	14	12	17
Thr	11	16	17	10	12	29	16	16	25	26
Trp	2	4	1	2	1	6	3	n.d.[c]	n.d.[c]	2
Tyr	5	5	11	4	4	2	4	11	6	3
Val	18	19	22	7	14	14	14	14	13	14
Total	189	185	287	160	151	257	158	197	213	188
Molecular weight	20,300	19,782	32,000	16,940	16,102	26,815	17,493	22,606	24,919	19,979
C-terminal	Arg	n.d.[c]	n.d.[c]	Ala	Ala	Pro	Thr	Ile	Leu	Thr
N-terminal	n.d.[c]	n.d.[c]	n.d.[c]	Acetyl-Ala	Acetyl-Ala	n.d.[c]	Acetyl-Ser	n.d.[c]	n.d.[c]	Acetyl-Met
References[f]	1	2	3	4,5	4	6,7	8-11	12	12	13,14

[a]Expressed as free acid but actually includes free acid and the relevant amide, since asparagine and glutamine were not determined as such.
[b]Uncertain whether residue is amide or free acid.
[c]n.d.: Not determined.
[d]American Type Culture 36 strain (AC 36 TNV).
[e]SV-C, Satellite associated with AC 36 TNV.
[f](1) Stubbs and Kaesberg 1964; (2) Chidlow and Tremaine 1971; (3) van Regenmortel 1967; (4) Tung and Knight 1972a; (5) Funatsu and Funatsu 1968; (6) Tung and Knight 1972b; (7) Miki and Knight 1968; (8) Anderer et al. 1960; (9) Anderer et al. 1965; (10) Tsugita et al. 1960; (11) Funatsu et al. 1964; (12) Uyemoto and Grogan 1969; (13) Harris and Hindley 1965; (14) Peter et al. 1972.

Table 8. Amino Acid Residues Per Subunit of Some Bacterial Virus Coat Proteins.

	Virus			
Amino Acid	Coliphage fd	Coliphage fr	Coliphage f2	Coliphage QB
Ala	9	16	14	15
Arg	0	4	4	7
Asn	0	10	11	8
Asp	3	4	3	7
Asx[a]		1		
Cys	0	2	2	2
Gln	1	5	6	8
Glu	2	6	5	5
Gly	4	9	9	7
His	0	0	0	0
Ile	4	6	8	4
Leu	2	5	8	12
Lys	5	7	6	7
Met	1	2	1	0
Phe	3	5	4	3
Pro	1	5	6	8
Ser	4	11	13	9
Thr	3	9	9	12
Trp	1	2	2	0
Tyr	2	4	4	4
Val	4	16	14	13
Total	49	129	129	131
Molecular weight	5,168	13,736	13,710	14,037
C-terminal	Ser	Tyr	Tyr	Tyr
N-terminal	Ala	Ala	Ala	Ala
References[b]	1	2	3	4

[a]Uncertain whether aspartic acid or asparagine.

[b](1) Asbeck et al. 1969; (2) Wittmann-Liebold and Wittmann 1967; (3) Weber and Konigsberg 1967; (4) Konigsberg et al. 1970.

tidase A when they are carboxylterminal amino acids, Arg, Asp, Cys, Glu, Gly, and Pro when in the penultimate position tend to decrease the rate of cleavage of C terminal amino acids that are normally readily released.

Carboxypeptidase B is effective mainly in the release of basic C terminal amino acids such as Arg and Lys.

Carboxypeptidase C catalyzes the hydrolytic cleavage of almost any C terminal residue, including Pro, although it is inefficient with such combinations as Pro-Pro and Pro-Gly, and also may not function on large polypeptides.

Carboxypeptidase Y catalyzes the release of most amino acids, including proline, from the C terminals of peptides and proteins (Hayashi et al. 1973). Glycine and aspartic acid may be released more slowly than other

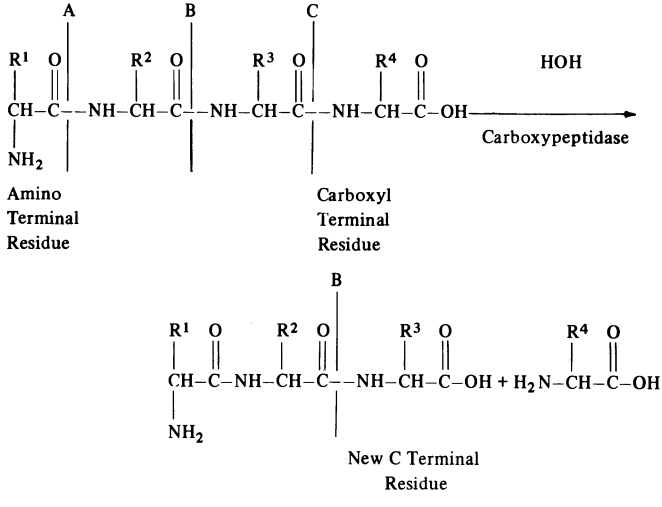
Table 9. Electrophoretic Isoelectric Points of Some Viruses.

Virus	Isoelectric Point pH	Reference
Alfalfa mosaic	4.6[a]	Lauffer and Ross 1940
Influenza A (PR8)	5.3	Miller et al. 1944
Shope papilloma	5.0	Beard and Wyckoff 1938 Sharp et al. 1942
Southern bean mosaic	5.9	MacDonald et al. 1949
Tobacco mosaic	3.5	Eriksson-Quensel and Svedberg 1936
Tomato bushy stunt	4.1	MacFarlane and Kekwick 1938
T_2 bacteriophage	4.2	Sharp et al. 1946
Turnip yellow mosaic	3.8	Markham and Smith 1949
Vaccinia	4.5	Beard et al. 1938
Wild cucumber mosaic	6.6	Sinclair et al. 1957

[a]Determined from minimum solubility rather than from electrophoretic measurements.

amino acids. The enzyme has the advantage of retaining activity in 6 M urea, which makes it possible to use it in structural studies on proteins whose C terminals are not exposed unless they are treated with chain-unfolding reagents such as urea.

The reaction of a peptide with carboxypeptidase A is illustrated by the following in which the R groups represent the side chains of common amino acids (such as H, CH_3, benzyl, and so on). The peptide bond at C is split and then, if conditions are favorable, the one at B.

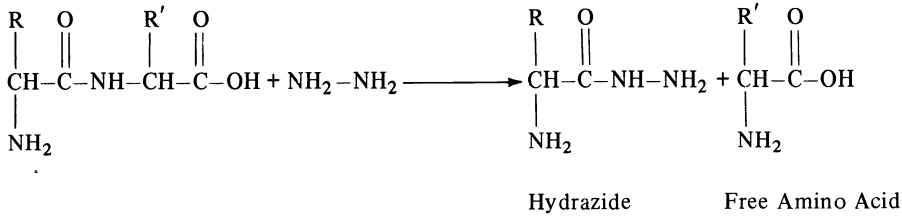


Another technique for determining the nature of C terminal amino acid residues is that employing hydrazinolysis (Akabori et al. 1956).

As shown by the following equation, in the hydrazinolysis reaction only the C terminal amino acid comes out as the free amino acid. All others are converted to hydrazides, which have different solubilities than the free amino acids and can be separated by extraction with appropriate organic solvents. The free amino acids can be identified by forming the dinitrophenyl derivatives and subjecting them to two-dimensional chromatography on paper. Elution of the spots and examination in the spectrophotometer provide quantitative values, which, after correction for the significant destruction that occurs during the hydrazinolysis step, are a measure of the C terminal end groups present.

Improved yields by use of a catalyst in the hydrazinolysis step and qualitative and quantitative determination of the free amino acids released by means of column chromatography enhance the usefulness of this technique (see Schroeder 1972).

The hydrazinolysis reaction may be illustrated with a dipeptide as follows:



The carboxypeptidase method has been singularly successful with TMV and strains, but has been disappointing with other viruses. For example, when applied to potato virus X, cucumber viruses 3 and 4, southern bean mosaic, tomato bushy stunt, and tobacco ringspot viruses, small, equivocal amounts of several amino acids were released from which no safe conclusions regarding the C terminals or numbers of subunits could be drawn (Knight 1955).

Three possible reasons can be suggested for this result: (1) The C terminal may contain sequences that are incompatible with the specificity of carboxypeptidase A. (2) More vigorous treatment (for example, more enzyme and/or higher temperature) may be needed. (3) The C terminal may be sterically unavailable to the enzyme. The following examples illustrate each of these situations.

C terminal proline is not cleaved by carboxypeptidase A. Potato virus X was found by the hydrazinolysis procedure (Niu et al. 1958; Miki and Knight 1968) to have a proline residue in the C terminal position, thus explaining the negative result with carboxypeptidase A.

Cucumber viruses 3 and 4 when treated with carboxypeptidase A at an enzyme:substrate ratio of about 1:400 at 25° yielded small amounts of several amino acids; and, while alanine seemed to be the major split product, even its concentration was so low as to make the result equivocal (Knight 1955). However, treatment at an enzyme:substrate ratio of 1:25 and at 37° clearly revealed alanine as the C terminal residue and threonine and serine as probable adjacent amino acids (Tung and Knight 1972a). The kinetics of the reaction are shown in Figure 10.

Two cases will serve to illustrate the steric hindrance possibility. No

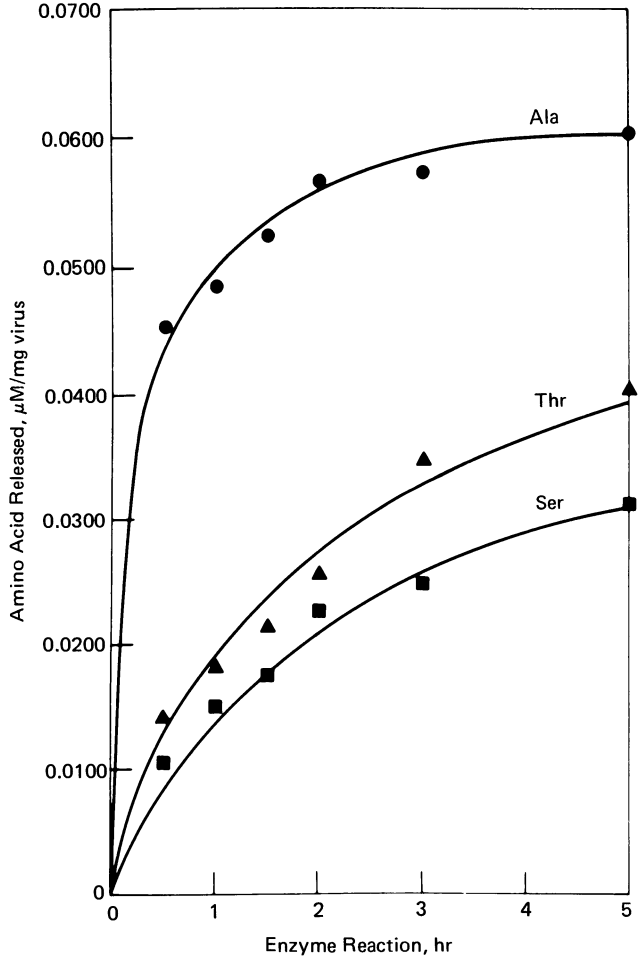
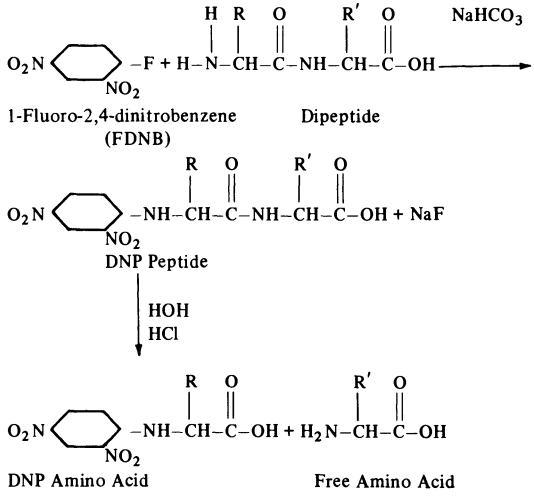


Fig. 10. Release of amino acids from Czech cucumber virus 4 by the action of carboxypeptidase A at an enzyme-substrate ratio of 1:25 at 37°. (From Tung and Knight 1972a.)

free amino acids were produced when intact turnip yellow mosaic virus was treated with carboxypeptidase, but when the isolated protein was employed, four different amino acids were released (Harris and Hindley 1961). From quantitative rate studies of the release, it was found that threonine was the C terminal amino acid, and that after removal of this, carboxypeptidase catalyzed the release from the peptide chain in succession—Ser, Thr, Val, and Asp. Another interesting case is that of the nitrous acid mutant TMV-171 (Tsugita and Fraenkel-Conrat 1960), from which carboxypeptidase caused the release of three amino acids, but from whose isolated protein the enzyme released about 15 residues.

There are several methods for determining the amino terminal (N terminal) residue of proteins and peptides, most of which, however, depend on the presence of an unsubstituted amino group. The two methods that have been used most in determining N terminal amino acid residues of viral proteins are (1) the Sanger fluorodinitrobenzene (FDNB) method (Sanger 1945, 1949; see also Knight 1964) and (2) the Edman phenylisothiocyanate (PTC) method (Edman 1950a, 1950b, 1956; see also Knight 1964).

The principle of the FDNB method is that the free amino group of the N terminal residue in a peptide or protein reacts with FDNB in mildly alkaline solution to give a *N*-dinitrophenyl (DNP) substituted residue. Upon acid hydrolysis, the DNP group remains attached to the N terminal amino acid in most cases, although some hydrolytic cleavage occurs with all DNP amino acids, being most severe with proline, glycine, and cysteine. The formation of the DNP derivative of a dipeptide and its subsequent hydrolysis can be illustrated as follows:



This general formulation becomes specific when R and R′ are replaced with H, CH_3, C_6H_5-CH_2, or other amino acid side chains.

The DNP amino acids are mainly extractable by ether and can thus be readily separated from nonterminal free amino acids, after which they are identified by paper chromatography and comparison with chromatograms of standard DNP amino acids. A standard chromatogram is given in Figure 11. The yellow color of DNP amino acids makes them easily located on chromatograms, and they can be quantitatively extracted and their concentrations determined from their light absorption in a spectrophotometer at 360 nm.

A procedure similar to the DNP method employs 1-dimethylaminonaphthalene-5-sulfonyl chloride ("dansyl" chloride) as the reagent. Dansyl (DNS) derivatives of the amino acids are very resistant to acid hydrolysis and show an intense yellow fluorescence that enables them to be detected at about 1 percent of the concentration needed for DNP amino acids. DNS derivatives can be identified by either electrophoresis or chromatography (see Bailey 1967; Gray 1972). The use of this technique is

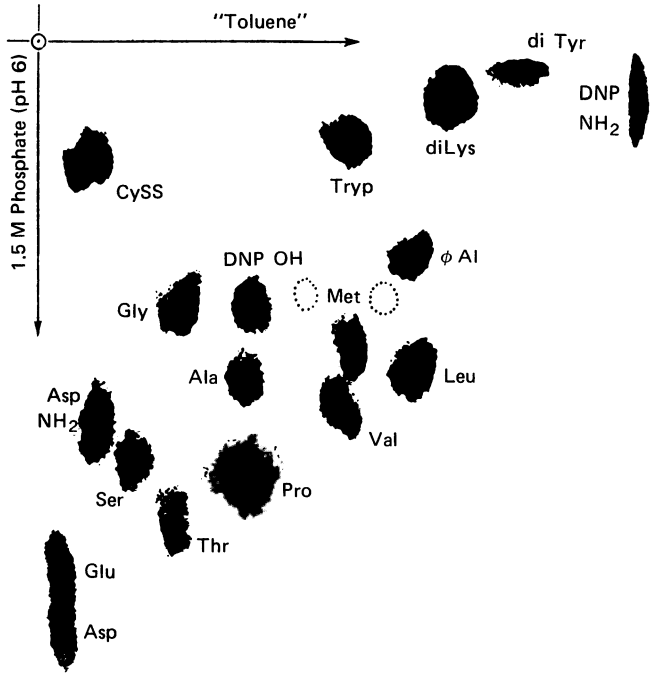
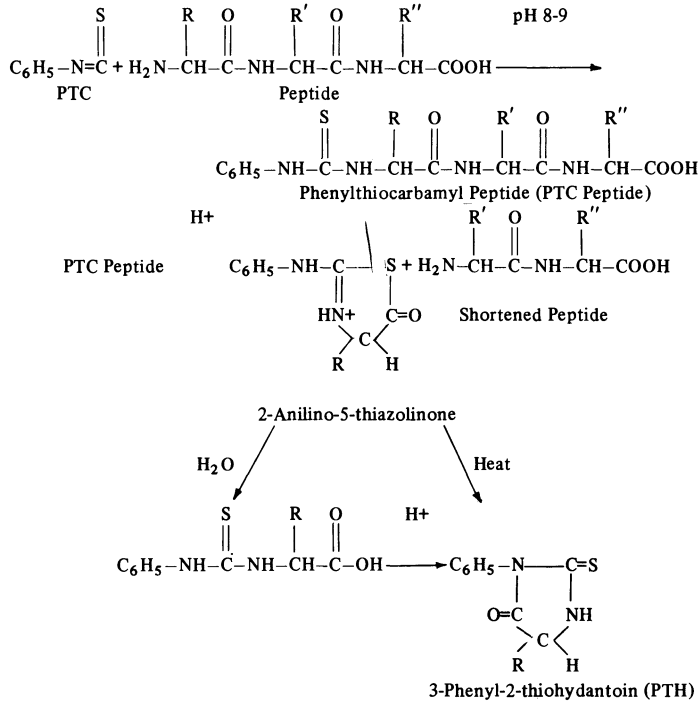


Fig. 11. Two-dimensional chromatogram of a mixture of 16 DNP-amino acids, dinitrophenol (DNPOH) and dinitroaniline (DNP NH_2). Approximately 0.02 μM of each was applied. (Fraenkel-Conrat et al. 1955.)

nicely illustrated by its application to the polypeptides of reovirus (Pett et al. 1973).

The major reactions of the Edman degradation, also called the phenylisothiocyanate (PTC) reaction, resemble the FDNB procedure in that an organic radical is first coupled to the protein at the N terminal in mildly alkaline solution and then the substituted N terminal amino acid is cleaved from the rest of the protein by treatment with acid. A milder acid treatment is used in the PTC method than in the FDNB procedure; hence, in contrast to the latter, the residual protein, shortened by one amino acid residue, is available for further stepwise degradative analysis. As in the case of the FDNB method, the procedure is applicable to peptides as well as to proteins. The chemical steps of the PTC reaction when applied to a peptide can be represented as follows:



As shown in the reaction scheme, the N terminal amino acid emerges as a 3-phenyl-2-thiohydantoin (PTH) derivative. The PTH derivative is extracted from the reaction mixture with an organic solvent in which the protein or peptide residue is insoluble. The quantity of PTH derivative can be estimated by reading its absorption in a spectrophotometer at the maximum (usually between 260 and 275 nm) and comparing the result with that

of an appropriate standard. Identification of the amino acid present as a PTH derivative can sometimes be made by paper chromatographic comparison with the standard PTH amino acids (Sjöquist 1953), or the PTH derivative can be hydrolyzed in acid to yield free amino acid, which then can be identified by any standard method for amino acid analysis. Various modifications of this procedure are used, including the substitution of potassium cyanate for phenylisothiocyanate (Stark and Smyth 1963; Stark 1972).

The methods just described for the determination of N terminal amino acid depend on the presence of a free amino group on the N terminal residue. However, it appears that many viral proteins have acetylated N terminal amino acids (see Table 10). Two methods have been employed in analyzing such N terminals:

1. The isolated viral protein is digested with pepsin or trypsin and the resulting peptides are fractionated on a strongly acidic cation exchange column. The peptide containing the acetylated amino acid as its N terminal will generally be least basic owing to the neutralization of its N terminal amino group by the acyl substituent. Therefore this peptide elutes from the column in one of the earliest fractions; it can be detected by the Folin colorimetric procedure and analyzed more or less readily by sequencing procedures (see Sec. IIIA, 2d). This approach was used successfully in identifying qualitatively the N terminal amino acids of TMV and cucumber virus 4 coat proteins (Narita 1958, 1959).

2. In the case of viral proteins acetylated at the N terminal, the amount of this terminal amino acid (and hence the molecular weight of the protein) can be estimated from the quantity of acetic acid released upon acid hydrolysis of the protein. The acetic acid can be determined readily by gas chromatography. Thus subunit molecular weights, but not the identity of the acetylated amino acids, were determined for tobacco mosaic and potato X virus proteins (Miki and Knight 1968). Some examples of the terminal amino acid residues found in some viruses are given in Table 9.

c. Protein Subunits

In 1952, Harris and Knight treated TMV with carboxypeptidase and found that more than 2,000 threonine residues were released from each mole of virus. The actual value from repeated determinations (Harris and Knight 1955) was about 2,320. (This figure is based on the now commonly used molecular weight for TMV of 40×10^6. The paper just cited gives a value of 2,900 based on a molecular weight for TMV of 50×10^6.) The key point, however, is that these results indicated that the TMV protein consists of over 2,000 polypeptide chains (protein subunits), and since these peptide chains all terminate in threonine it was correctly assumed, as subsequent data have shown, that they represent identical subunits. The alternative possibilities of a few chains ending in polythreonyl units or a

Table 10. The Protein Subunits of Some Viruses.

Virus	No. of Constituent Proteins	Approximate Molecular Weight of Subunits	N-Terminal	C-Terminal	Ref.[a]
Alfalfa (lucerne) mosaic	1	25,000	Ac-Ser	Arg	1,2
Broad bean mottle	1	20,500		Ala	3
Bromegrass mosaic	1	18,000		Arg	4
Cucumber 4	1	16,000	Ac-Ala	Ala	5,6,6a
Cucumber mosaic	1	24,000			7
Potato X	1	27,000	Ac-X[b]	Pro	8
Shope papilloma	1	40,000		Thr	9
Southern bean mosaic	1	30,000		Ser	10
Sowbane mosaic	1	19,000		Lys	11
Tobacco mosaic	1	17,500	Ac-Ser	Thr	12,13
Tobacco necrosis	1	30,000	Ala	Met	14
Tobacco necrosis satellite	1	20,000	Ala	Ala	15
Tobacco rattle	1	24,000			16
Tomato bushy stunt	1	41,000		Leu	17,18
Turnip yellow mosaic	1	20,000	Ac-Met	Thr	19
White clover mosaic	1	22,500			8

Adeno	9	7,500–120,000	20
Coliphage f2	2	14,000 and 39,000	21
Coliphage ØX174	4	5,000– 48,000	22
Coliphage T4	~28	11,000–120,000	23
Herpes simplex	~24	25,000–275,000	24
Influenza	~7	25,000– 94,000	25–27
Mouse Elberfeld	4	7,300– 33,000	28
Newcastle disease	~6	41,000– 74,000	29
Mouse mammary tumor	5	23,000– 90,000	30
Polio	4	6,000– 35,000	31
Polyoma	6	13,000– 43,000	32
Reovirus	~7	34,000–155,000	33
Rubella	~3	35,000– 62,000	34
Simian 40	6	9,300– 42,000	32
Vaccinia	~30	8,000–200,000	35

[a](1) Hull et al. 1969; (2) Kruseman et al. 1971; (3) Miki and Knight 1965; (4) Stubbs and Kaesberg 1964; (5) Narita 1959; (6) Niu et al. 1958; (6a) Tung and Knight 1972a; (7) Van Regenmortel 1967; (8) Tung and Knight 1972b; (9) Kass 1970; (10) Ghabrial et al. 1967; (11) Kado 1967; (12) Narita 1958; (13) Harris and Knight 1955; (14) Lesnaw and Reichmann 1969; (15) Reichmann 1964; (16) Offord and Harris 1965; (17) Tung and Knight, unpublished (from gel electrophoresis); (18) Niu et al. 1958; (19) Harris and Hindley 1961; (20) Maizel et al. 1968a, 1968b; (21) Hohn and Hohn 1970; (22) Burgess and Denhardt 1969; (23) Laemmli 1970; (24) Spear and Roizman 1972; (25) Compans et al. 1970; (26) Skehel and Schild 1971; (27) Schulze 1972; (28) Rueckert et al. 1969; Stoltzfus and Rueckert 1972; (29) Mountcastle et al. 1971; (30) Nowinski and Sarkar 1972; (31) Jacobson et al. 1970; (32) Hirt and Gesteland 1971; (33) Pett et al. 1973; (34) Vaheri and Hovi 1972; (35) Sarov and Joklik 1972.

[b]X = N-terminal amino acid residue unknown.

single huge polypeptide possessing threonyl side chains on ω carboxyl groups of aspartic or glutamic acid residues were eliminated by appropriate tests (Harris and Knight 1955). Also, application of the newly developed Akabori hydrazinolysis method (1956) confirmed the original conclusion that the virus protein consists of many peptide chains, each terminating in a single threonyl residue (Braunitzer 1954; Niu and Fraenkel-Conrat 1955).

There are several ways of calculating numbers of subunits in a viral protein from terminal amino acid data obtained as described in the preceding section. One of them is illustrated here, with the use of data from TMV analyses.

The molecular weights of TMV and threonine are 40×10^6 and 119, respectively. These molecular weights may be expressed in any units desired for the purposes of calculating relationships between TMV and its C terminal threonine. Thus the same end result will be obtained whether calculations are made using grams, milligrams, or micrograms. If micrograms are used, for example, the weight of one TMV particle (particle or molecular weight) is expressed as 40×10^6 μg, representing 1 μM of TMV. (The absolute weight of one TMV particle is, of course, the particle or molecular weight in grams divided by the number of particles in one mole, that is, by Avogadro's number. Thus the actual weight of one particle of TMV, assuming a molecular weight of 40×10^6, is 6.64×10^{-17} g.)

From 10^4 μg of TMV there were released by carboxypeptidase A 62 μg of threonine. Then

$$\text{Micromoles of threonine released} = \frac{\mu\text{g of threonine found}}{\text{molecular weight of threonine}}$$

$$= \frac{62}{119} = 0.52$$

$$\text{Micromoles of TMV used} = \frac{10^4}{40 \times 10^6} = 2.5 \times 10^{-4}$$

If 2.5×10^{-4} μM of TMV yielded 0.52 μM of threonine, 1 μM of TMV would yield $1 / (2.5 \times 10^{-4}) \times 0.52 = 2{,}080$ μM of threonine. Therefore, there must be 2,080 polypeptide chains (protein subunits) in the TMV particle. Since the virus is 95 percent protein, the molecular weight of each protein subunit is $(0.95 \times 40 \times 10^6) / 2080 =$ about 18,000.

There are many simple viruses like TMV that consist of a strand of nucleic acid ensheathed in a multisubunit protein coat. However, the large, tailed phages and many of the larger animal viruses have more than one species of protein in their particles. In these cases, it is common for each species of protein to be made up of identical subunits. Thus the major head protein of the T-even coliphages consists of about 2,000 identical subunits, each with a molecular weight of 40,000, while another protein, the tail sheath protein, is composed of 144 subunits, each of two species of

polypeptide, and so on (Mathews 1971). It is possible in some cases at least to isolate each species of protein from a virus particle and subject it to structural analysis, that is, analyze for amino acid content, do end group analyses, and so on. Often, however, it is useful just to determine the number of different polypeptide species present in the virus and their approximate molecular sizes. This can be done by applying the techniques of electrophoresis in acrylamide gel (Shapiro et al. 1967, 1969; Weber and

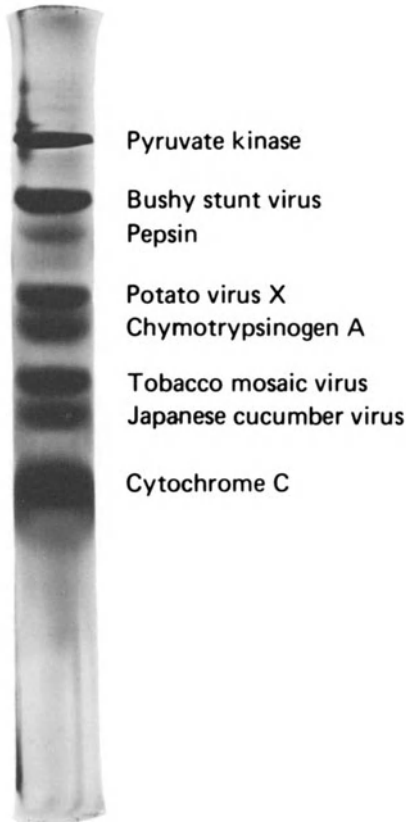


Fig. 12. Sodium dodecyl sulfate-polyacrylamide gel electrophoregram of some proteins ranging in molecular weight from 11,700 (cytochrome c) to 57,000 (pyruvate kinase). Electrophoretic migration, which was in 10 percent gel, is from top toward bottom in the illustration. The bands are visualized by staining in 0.25 percent Coomassie brilliant blue.

Osborn 1969; Dunker and Rueckert 1969) or gel chromatography (also called gel filtration) in agarose (Fish et al. 1969) to dissociated whole virus or to the protein fractions isolated from purified virus by one of the methods described in Sec. IIIA, 1.

In both techniques the molecular weights are determined by comparison of the migration rates of viral polypeptides with those of standard proteins whose molecular weights have been established by various means.

Figure 12 illustrates the migration of several plant virus proteins and standard proteins in SDS—10 percent acrylamide gels. The molecular weights assumed for the standard proteins and those calculated for the viral proteins shown in the gel of Figure 12 are pyruvate kinase, 57,000; tomato bushy stunt virus protein, 41,000; pepsin, 35,000; potato virus X protein, 27,000; chymotrypsinogen A, 25,700; tobacco mosaic virus protein, 17,500; Japanese cucumber virus 3 protein, 16,000; and cytochrome C, 11,700.

All of the plant viruses used in the illustration just given are characterized by a single species of polypeptide comprising the subunits of the viral coat protein. The electrophoresis of the polypeptides of influenza virus, which contains several different species, is illustrated in Figure 13. In this case, the whole virus was dissociated by treatment with SDS and the mixture was applied to the gel for electrophoresis. Duplicate gels are shown in order to illustrate how polypeptides and glycopolypeptides can

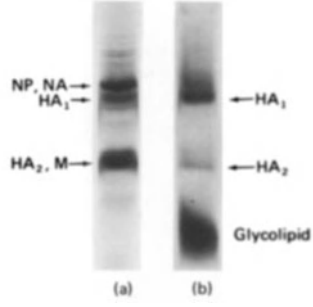


Fig. 13. Polyacrylamide gel electrophoregram of PR8 influenza virus dissociated in 1 percent sodium dodecyl sulfate and run on a 6 percent gel. Gel (*a*) was stained with Coomassie brilliant blue to reveal protein bands, and gel (*b*) was stained with *p*-rosanilin to detect carbohydrate (glycoprotein, two upper bands; glycolipid, lower band).

Abbreviations: NP, nucleoprotein (nucleocapsid subunit); NA, neuraminidase; HA_1, large hemagglutinin component; HA_2, small hemagglutinin component; M, membrane protein of viral envelope.

be distinguished after electrophoresis by application of different stains.

An extraordinarily useful modification of gel electrophoresis is the use of thin gel slabs that permit the side-by-side comparison of many samples (Reid and Bieleski 1968; Studier 1973). Location of protein bands (the procedure is also applicable with modifications to RNAs) is usually accomplished by autoradiography of the dried gels, appropriate isotopes having been previously introduced into the system under investigation. Quantitative distinctions between bands can be enhanced more readily in autoradiography than in the gel staining techniques simply by varying the length of time the recording film is exposed to the gel. Examples of such gels are shown in Figure 13A, which records the proteins extracted from *E. coli* cells infected with various combinations of coliphage P2 and its satellite P4 as well as certain mutants (Barrett and Calendar 1974; Lengyel et al. 1973, 1974). An excellent example of the application of the slab gel technique to the analysis of proteins in an animal virus system is the study by Honess and Roizman (1973) of herpes simplex proteins.

Some caution needs to be exercised with respect to molecular weight values for viral or other proteins obtained by SDS polyacrylamide gel electrophoresis. It has often been assumed that the rate of migration of polypeptides in SDS acrylamide gels depends solely on their molecular size. This assumption appears valid for many proteins. However, a rigorous application of the technique to plant virus proteins (Tung and Knight 1972a, 1972b, 1972c) indicates that the electrophoretic migrations of proteins of similar size in SDS polyacrylamide gels is a closely related function of their molecular weights only when the macromolecules under investigation have the same hydrodynamic shape and charge-to-mass ratio. This situation exists only when standard and test proteins react with SDS in a strictly comparable manner. The data summarized in Table 11 illustrate this point.

The values listed in the first column of the table, which were determined by amino acid analysis and peptide mapping as described earlier, represent the most accurate figures available. They probably deviate from the actual molecular weights by not more than one or two amino acid residues (± 100–200 daltons) and less than that for TMV whose complete amino acid sequence is known. A comparison of the molecular weight determined by other methods with that in the first column shows a good agreement for TMV (± 10 percent accuracy is usually ascribed to the gel electrophoresis and ± 7 percent accuracy is associated with gel chromatography results).

However, focusing on the SDS acrylamide gel values for all of the viruses listed in the table, it is apparent that close agreement with the actual values (column 1) was observed only with TMV and Japan CV3 proteins. The values indicated by gel electrophoresis are spuriously low for Berkeley CV3, Berkeley CV4, and Czech CV4 proteins. It seems likely that

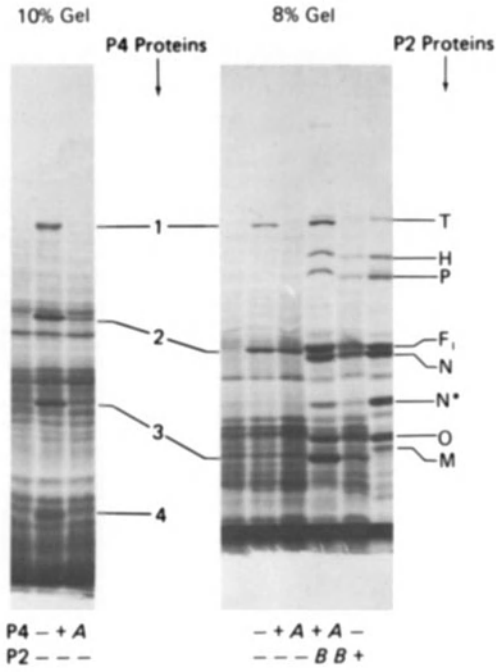


Fig. 13A. Use of slab gel electrophoresis and autoradiography to detect proteins made after infection of *Escherichia coli* bacteria with various combinations of coliphages P2 and P4 and mutants A and B. (Courtesy K. Barrett.)

E. coli C was irradiated with ultraviolet light in order to decrease the synthesis of host proteins. The cells were then infected with P2, P4, or with both together, and labeled with ^{14}C-reconstituted algal protein hydrolysate from 70 to 72 min after infection (*left panel*) or from 40 to 60 min after infection (*right panel*). The incorporation was stopped by adding an excess of cold amino acids and pouring the cells in ice. The cells were collected by centrifugation and lysed by holding in boiling water for 2 min in tris buffer at pH 6.8 containing SDS and mercaptoethanol (Laemmli 1970). The labeled proteins were analyzed in SDS polyacrylamide slab gels. The discontinuous gel system of Laemmli and Maizel as described by Laemmli (1970) was used with a 5 percent stacking gel and an 8 or 10 percent resolving gel. The slab gel apparatus was that of Studier (1973). The gels were dried for autoradiography according to Maizel (1971) and autoradiograms were made with Kodak No-Screen x-ray film.

Symbols: –, no phage; +, wild-type phage as indicated at the left; A or B, phage which contains mutations in genes A or B.

Table 11. Molecular Weights of Coat Protein Subunits of Tobacco Mosaic Virus and of Some Isolates of Cucumber Viruses 3 and 4 Determined by Different Methods.[a]

	Method of Determination			
Virus	Amino Acid Analyses and Peptide Mapping	C-terminal Analysis	SDS-Polyacrylamide Gel Electrophoresis	Agarose Gel Chromatography in Guanidine Hydrochloride
Tobacco mosaic	17,500	18,000	18,000	16,500
Berkeley CV3	17,100	10,100	14,200	16,000
Japan CV3	17,100	25,000	16,000	16,000
Berkeley CV4	16,100	13,300	14,200	16,000
Czech CV4	16,100	16,700	14,200	16,000

[a]Adapted from Tung and Knight 1972a.

these proteins retain enough tertiary structure in the presence of SDS to cause them to migrate at anomalous rates with respect to the standard proteins (and with respect to the proteins of TMV and Japan CV3). Consequently, as judged by SDS gel electrophoresis, the proteins of Berkeley CV3 and Japan CV3 appear to have significantly different molecular weights when, in fact, they are the same, and, conversely, the proteins of Berkeley CV3 and Berkeley CV4 appear to have the same molecular weight when, in fact, they are substantially different.

From these and other data, Tung and Knight have concluded that, as might be expected, the most accurate procedure for determining the molecular weights of viral and other polypeptides is to add up the weights of the constituent amino acid residues. In practice, this means that one determines the minimum number of residues in (and hence a minimum molecular weight of) the polypeptide from careful amino acid analyses and then determines the factor by which the minimum value must be multiplied to give the actual value. This factor is the integral number nearest to the quotient obtained by dividing the molecular weight estimated by gel electrophoresis or gel chromatography by the minimum molecular weight based on amino acid analysis. Alternatively, the factor can be deduced by comparing the number of peptides found on a map of the tryptic digest of the protein with the number expected from the arginine and lysine residues in the minimum molecular weight unit.

Obviously, the less accurate molecular weight values for viral polypeptides (protein subunits) obtainable by gel electrophoresis and gel chromatography are often sufficient and may be much more convenient to obtain since they do not require the chemically homogeneous product required for reliable amino acid analyses. Thus the choice of method for determining molecular weights of viral proteins will doubtless depend on conveni-

ence and degree of accuracy sought. In addition, it is clear that information other than molecular weight can be obtained by the various procedures employed, for example, numbers of different polypeptide species present, presence or absence of conjugated carbohydrate moieties, nature of terminal groups of the polypeptide chain, and so on.

Information about the numbers, size, and composition of polypeptides associated with each virus is still fragmentary. However, some data of this sort appear in Table 10.

It can be seen from the data in Table 10 that while there are some large viral polypeptides, most of them fall in the range of 14,000–50,000. As might be expected, the larger, morphologically complex viruses contain several species of polypeptides including some of the larger ones. The rather common occurrence of acylated N terminals is also illustrated.

d. Amino Acid Sequences

Most proteins contain basic amino acids such as arginine and lysine scattered along the length of the peptide chain. This fact, coupled with the marked specificity of the enzyme trypsin for bonds next to basic amino acids, provides a means for cleaving long peptide chains into more readily analyzed fragments.

A convenient survey of the peptides can be made by combining paper electrophoresis and chromatography in a "mapping" procedure. This is illustrated by the diagram of Figure 14, which shows a peptide map obtained after digestion of TMV protein with trypsin. However, the amounts of individual peptides in map spots seldom exceed 200 μg, whereas milligram amounts are usually required for sequential analyses. Therefore, countercurrent extraction or ion exchange chromatography is usually done to separate tryptic peptides for sequential analysis. The separation of the tryptic peptides from TMV protein by ion exchange chromatography is illustrated by the elution diagram shown in Figure 15.

The next step is to determine the amino acid sequences of each peptide. These steps can be illustrated by taking the peptide designated as 11 in Figure 15 and following the sequence determination made by Ramachandran and Gish (1959).

The purity of an aliquot of this fractionated peptide was checked by the mapping procedure. The spot labeled 11 in Figure 14 was the only major spot observed. N terminal analysis by the DNP method indicated that glycine was the N terminal residue. Analysis of the rest of the peptide by acid hydrolysis, formation of DNP derivatives, and so on (instead of the DNP procedure, it is also convenient to subject the hydrolysate to analysis directly in the automatic amino acid analyzer) showed amino acids present in the following molar proportions: Arg 1.00, Asp 1.14, Gly 0.98, Ser 1.00, Thr 1.00, Tyr 0.86. Hence the peptide is a heptapeptide with the formula Gly (Arg, Asp, Gly, Ser, Thr, Tyr). (In the formula the usual convention of

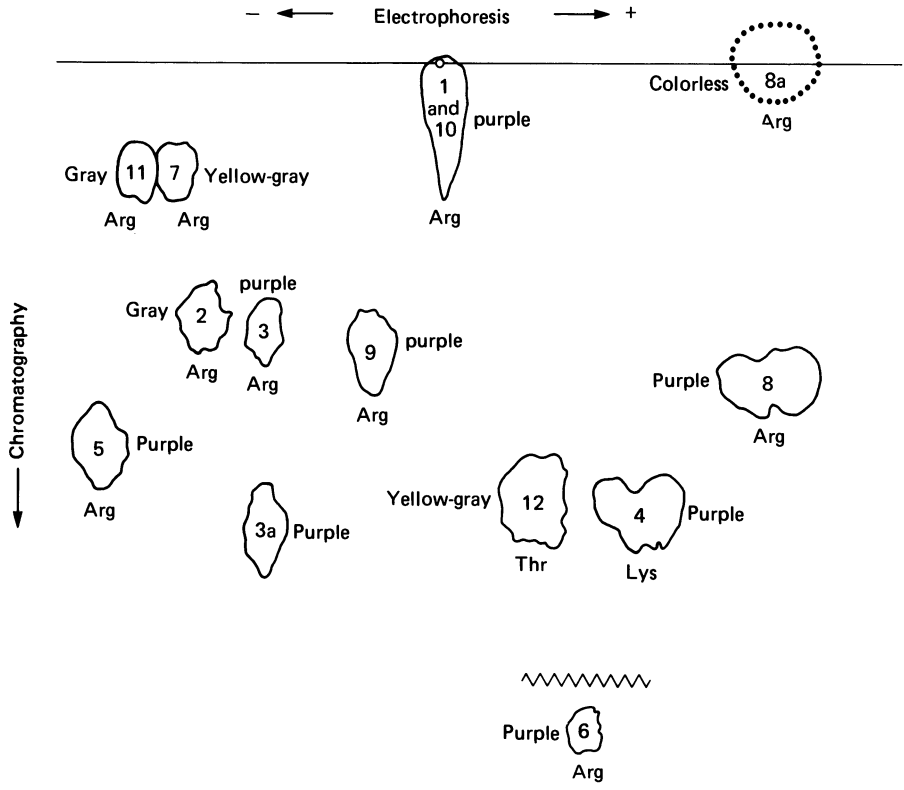


Fig. 14. Map of the peptides obtained by digestion of tobacco mosaic virus coat protein with trypsin. Adapted from Woody and Knight (1959) by deletion of four minor spots and addition of three major ones (tryptic peptides 1, 6, and 10) not shown on the original map. The latter were largely removed by precipitation at pH 4.5 prior to mapping in the experiments of Woody and Knight. Their migration in the mapping procedure was subsequently determined when the individual peptides became available. Peptide 6 travels much farther down the sheet than shown here, and the wavy line above it indicates that it has been brought up to a point more conveniently included in the diagram. Solid lines indicate ninhydrin-reactive spots that gave the colors indicated, and dotted lines signify a ninhydrin-negative but starch-iodine positive spot. The abbreviations for the amino acids—Arg, Lys, and Thr—indicate the nature of the C-terminal amino acid residue in a given peptide. See Table 12 for compositions of the peptides, which are numbered in order of their occurrence from the N-terminal to the C-terminal.

the protein chemists is used in which the amino acids whose sequences are unknown are placed in parentheses.)

Another portion of peptide 11 was treated with the enzyme leucine aminopeptidase, and aliquots were removed at various time intervals and analyzed by the DNP method. (Leucine aminopeptidase catalyzes hydrolytic cleavage of amino acids in a stepwise fashion from the N terminal end

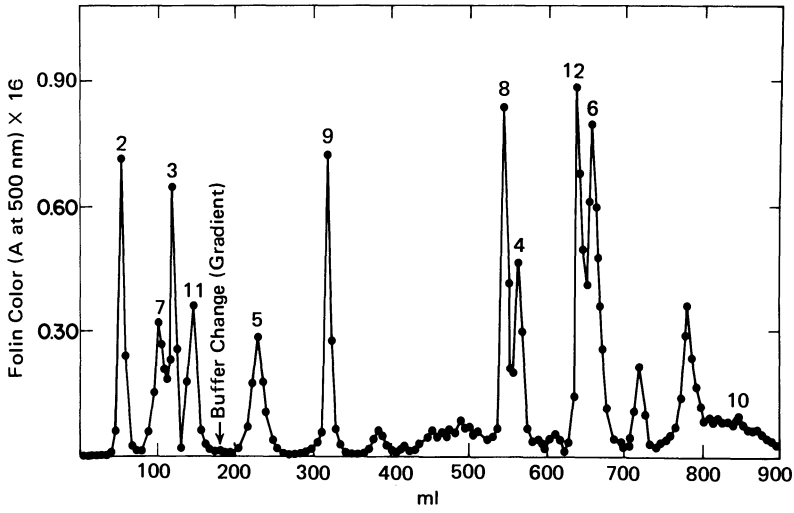


Fig. 15. Separation of tryptic peptides of TMV protein, after removal of material insoluble at pH 4.5 (mostly peptide 1) by passage through a column of Dowex 1-X2. The peptides were detected by their reaction with the Folin reagnet. The numbers are those of the tryptic peptides as listed in Table 12. (From Tsugita and Fraenkel-Conrat 1962.)

of peptide chains.) The results obtained are shown in Figure 16. These results indicate an N terminal order of Gly-Thr-Ser followed by asparagine and tyrosine in unknown order. It will be noted that the enzymatic degradation of the peptide revealed that one of the residues was asparagine rather than aspartic acid. Acid hydrolyses always yield the free acids rather than the amides, so that enzymatic hydrolyses are important in distinguishing between aspartic acid and asparagine and between glutamic acid and glutamine.

Another portion of peptide 11 was hydrolyzed with the enyzme chymotrypsin. Test analyses made by paper chromatography of portions of digest and by use of various indicator sprays indicated that the peptide was split rapidly into two peptides. One of these gave a positive test for arginine and the other a positive color reaction for tyrosine. Amounts of each peptide sufficient for analyses could be obtained by paper chromatography of the chymotryptic digest followed by elution of the separated peptides; a small strip of the chromatogram was reserved for spraying with ninhydrin in order to locate the spots. One of the peptides was found by application of the DNP method to contain equimolar amounts of aspartic acid (asparagine before hydrolysis) and arginine, the aspartic acid being N terminal. Hydrolysis of a portion of this peptide by leucine aminopeptidase yielded asparagine and arginine. Hence the peptide was Asn-Arg. A portion of the second peptide isolated from the chymotryptic digest of peptide 11 was

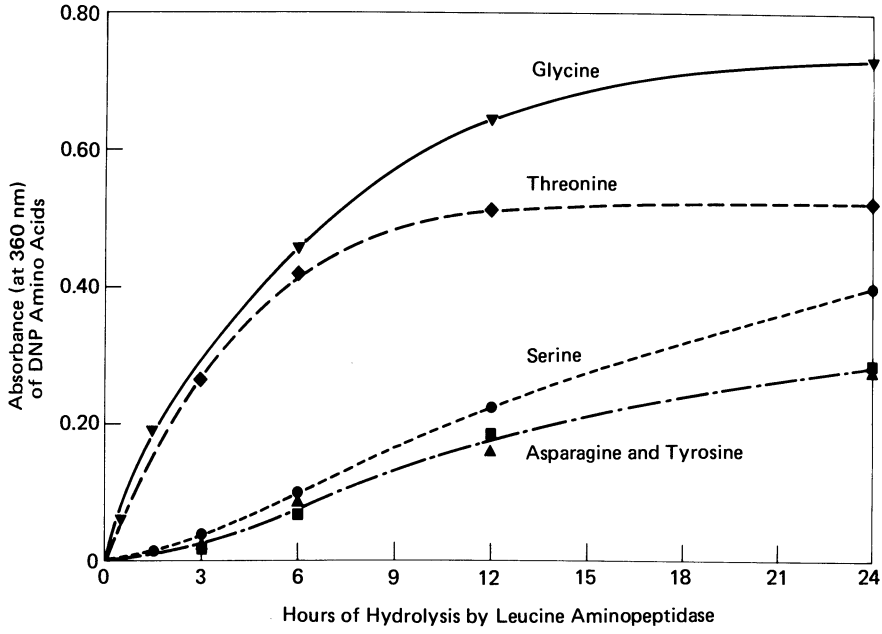


Fig. 16. Release of N-terminal amino acids from TMV tryptic peptide 11 by treatment with leucine aminopeptidase. The amino acids released at various times were identified as their DNP derivatives. (Adapted from Ramachandran and Gish 1959.)

treated with carboxypeptidase. Only tyrosine was released. Analysis of the remainder of the peptide by the DNP method revealed N terminal glycine and about equimolar proportions each of threonine, serine, and glycine. On the basis of these results and those obtained upon treating whole peptide 11 with leucine aminopeptidase, a sequence of Gly-Thr-Ser-Gly-Tyr could be assigned. Upon combination of the analytical results, the complete sequence for peptide 11 was found to be Gly-Thr-Ser-Gly-Tyr-Asn-Arg.

Twelve tryptic peptides were obtained from TMV protein and each was analyzed in a manner just outlined for peptide 11. The results are summarized in Table 12.

The final step in the primary structural analysis of TMV protein was to obtain "bridge peptides" whose sequences overlapped those of the peptides obtained by tryptic digestion. These were obtained by digesting portions of TMV protein with the chymotrypsin, pepsin, and subtilisin, and separating the resulting peptides by the same procedures used for the tryptic peptides. Analyses, or partial analyses, of the amino acid sequences of these new peptides provided information for linking the 12 tryptic peptides in the proper order to give the total sequence for the protein subunit. This procedure may be illustrated as follows.

Table 12. Peptides Obtained from TMV Protein by Tryptic Digestion.

Peptide Sequential Number	Number of Amino Acid Residues and Sequential Locations	Composition and Sequence	Color with Ninhydrin on Paper
1	41 (1-41)	Acetyl-Ser-Tyr-Ser-Ile-Thr-Thr-Pro-Ser-Gln-Phe-Val-Phe-Leu-Ser-Ser-Ala-Trp-Ala-Asp-Pro-Ile-Glu-Leu-Ile-Asn-Leu-Cys-Thr-Asn-Ala-Leu-Gly-Asn-Gln-Phe-Gln-Thr-Gln-Gln-Ala-Arg	Colorless
2	5 (42-46)	Thr-Val-Val-Gln-Arg	Gray
3	15 (47-61)	Gln-Phe-Ser-Gln-Val-Trp-Lys-Pro-Ser-Pro-Gln-Val-Thr-Val-Arg	Purple
3A	Same as 3, except for the preparative artifact of an N-terminal pyroglutamyl residue. See discussion by Gish (1960) of peptide K-0.66-A.		Purple
4	7 (62-68)	Phe-Pro-Asp-Ser-Asp-Phe-Lys	Purple
5	3 (69-71)	Val-Tyr-Arg	Purple

6	19 (72-90)	Tyr-Asn-Ala-Val-Leu-Asp-Pro-Leu-Val-Thr-Ala-Leu-Leu-Gly-Ala-Phe-Asp-Thr-Arg	Purple
7	2 (91-92)	Asn-Arg	Yellow-gray
8	20 (93-112)	Ile-Ile-Glu-Val-Glu-Asn-Gln-Ala-Asn-Pro-Thr-Thr-Ala-Glu-Thr-Leu-Asp-Ala-Thr-Arg	Purple
8A	Same amino acids as in 8. Exact structure unknown. Probably an N-terminal cyclic derivative of 8.		Colorless
9	10 (113-122)	Arg-Val-Asp-Asp-Ala-Thr-Val-Ala-Ile-Arg	Purple
10	12 (123-134)	Ser-Ala-Ile-Asn-Asn-Leu-Ile-Val-Glu-Leu-Ile-Arg	Purple
11	7 (135-141)	Gly-Thr-Gly-Ser-Tyr-Asn-Arg	Gray
12	17 (142-158)	Ser-Ser-Phe-Glu-Ser-Ser-Ser-Gly-Leu-Val-Trp-Thr-Ser-Gly-Pro-Ala-Thr	Yellow-gray

One of the peptides isolated from a chymotryptic digest of TMV protein was identified as Lys-Val-Tyr. There are two Lys residues in TMV protein, and one of them is in the tryptic peptide with a sequence -Lys-Pro-Ser (peptide 3 in Table 12). The other Lys is found in tryptic peptide 4 in a sequence ending in -Asp-Phe-Lys. This latter lysine must, therefore, be the one in the chymotryptic peptide, Lys-Val-Tyr. The only tryptic peptide with a Val-Tyr sequence is Val-Tyr-Arg. Hence the chymotryptic peptide bridges the two tryptic peptides listed in Table 12 as 4 and 5 and establishes their order in the TMV protein subunit.

By similar analyses, all of the tryptic peptides were located to give the total sequence of amino acids for the coat protein of common TMV and subsequently for other mutant strains. The sequences of four such strains are compared in Table 13.

The total number of amino acid residues in the coat proteins of many strains of TMV proved to be the same, 158. However, the coat proteins of some strains appear to reflect both additions and deletions in the viral genome (see Hennig and Wittmann 1972; Tung and Knight 1972a). Two amino acid deletions in the HR protein and their presumed location are indicated in Table 13. Admittedly, the relative evolutionary histories of these two strains are not known, and hence it can be argued that TMV was derived from an HR-like strain rather than the reverse. In that case, TMV protein would be viewed as possessing two additional amino acids rather than HR protein representing two deletions.

A comparison of the amino acid sequences of the fr, f2, and MS 2 bacteriophage coat proteins is shown in Table 14, and the sequence of the smallest known viral coat protein, that of fd phage, is given in Table 15 where it is compared with the protein of the closely related phage ZJ-2.

3. *Function of Viral Proteins*

The mass of most viruses is protein, much of which is located on the exterior of the virus particle. Thus situated, the protein comprises a coat or shell inside of which, or deeply embedded in which, lies the viral genetic material, the nucleic acid. Presumably, in the course of evolution, viruses whose genomes coded for a protein coat possessed survival value superior to those without such a structure. In any case, a noteworthy function of viral protein is the protection of the viral nucleic acid from destruction by nucleases or other degradative agents.

Another important function of viral protein is to mediate the process of infection, often determining the host specificity of a given virus. The basis for this action is that the first step in infection by many animal and bacterial viruses (but apparently not for plant viruses) is the attachment of a virus particle to a receptor site on a cell. A specific viral protein is involved in this attachment. In the case of tailed bacteriophages, the tail fibers, which

are protein, serve as specific attachment organs. Only phages whose tail fiber proteins have affinity for receptor sites on the bacterial envelope can attach and initiate infection. Spheroidal phages, such as the RNA-containing R17, f2, and fr phages, appear to possess a specific coat protein essential for initiation of infection even though they have no tails. Likewise, the coat proteins of animal viruses appear to be important in the capacity of these viruses to infect cells.

A striking example of the specificity that animal viral proteins can display is given by poliovirus. This virus when intact has a very restricted host range, namely, primate cells, and this restriction appears to be dependent on the specific affinity between poliovirus coat protein and receptor material on the primate cell surface (Holland 1964). However, when poliovirus RNA is used as the infectious agent, the host range of the virus is vastly expanded (for example, virus production occurs after intracerebral inoculations of mice, rabbits, guinea pigs, chicks, and hamsters) because the RNA gains entrance to cells by an inefficient, nonspecific mechanism. It should be noted that such "unnatural" infections are restricted because whole virus is produced in the cell initially infected by the RNA and whole virus can only attach productively to primate cells.

Viruses are good antigens. That is, when introduced into various animals either by injection or by infection they elicit the production of antibodies. These antibodies can react with viruses in a variety of immunologic and serologic ways (See vol. II, chap. 13 in Fenner 1968; Casals 1967; Matthews 1967). Viral proteins either alone or in some viruses as glycoproteins and lipoproteins are primarily responsible for such properties partly because they comprise a large part of the mass of virus particles and are exteriorly located, and especially because they are better antigens than other constituents of viruses.

Most of the larger and structurally more complex viruses contain enzyme constituents (these should be distinguished from the enzymes coded for by the viral genome but which do not become incorporated into the virus particles). Such enzymes are important protein constituents of viruses that have them. Functionally, they seem to fall mainly into two classes: enzymes that degrade cell envelope or membrane constituents (for example, phage lysozyme and influenzal neuraminidase) and those involved in viral nucleic acid synthesis (for example, the RNA transcriptase of reovirus and the RNA polymerase of Newcastle disease virus and the DNA polymerase called "reverse transcriptase" of Rous sarcoma virus) (Kozloff 1968; Webster 1970; Drzeniek 1972; Shatkin and Sipe 1968; Kingsbury 1972; Baltimore 1970; Temin 1970).

Protein kinases have been detected in the particles of several purified animal viruses, including numerous RNA tumor viruses, some viruses of the influenza and parainfluenza groups, vaccinia virus, and some herpes viruses (see Rosemond and Moss 1973). However, some viruses containing

Table 13. Sequence of Amino Acids in the Coat Proteins of Four Strains of Tobacco Mosaic Virus.[a]

	1	5	10	15
TMV[a]	Acetyl-Ser-Tyr-Ser-Ile-	Thr-Thr-Pro-Ser-	Gln-Phe-Val-Phe-Leu-	Ser-Ser-Ala-Trp-Ala-
D		-Ser-		-Val-
U2	Pro-	-Thr- -Asn-	-Tyr-	-Ala-Tyr-
HR	Acetyl-Ser-	-Asn- -Thr-Asn-Ser-Asn-	-Tyr-Gln-	-Phe-Ala-Ala-Val-Tyr-

	20	25	30	35
TMV	Asp-Pro-Ile-Glu-Leu-	Ile-Asn-Leu-Cys-	Thr-Asn-Ala-Leu-Gly-	Asn-Gln-Phe-Gln-Thr-
D		-Leu- -Val-	-Ser-Ser-	
U2	-Val-	-Ile- -Leu-	-Asn-Ala-	
HR	Glu-	-Thr-Pro-Met-Leu- -Gln-	—Val-Ser-	-Ser-Gln-Ser-Tyr-

	40	45	50	55
TMV	Gln-Gln-Ala-Arg-Thr-	Val-Val-Gln-Arg-	Gln-Phe-Ser-Gln-Val-	Trp-Lys-Pro-Ser-Pro-
D		-Thr- -Gln-	-Glu-	-Phe-
U2			-Ala-Asp-Ala-	-Ser-
HR	-Ala-Gly-	-Asp- -Arg-	-Asn-Leu-Leu-Ser-Thr-	Ile-Val-

	60	65	70	75
TMV	Gln-Val-Thr-Val-Arg-	Phe-Pro-Asp-Ser-	Asp-Phe-Lys-Val-Tyr-	Arg-Tyr-Asn-Ala-Val-
D		-Gly-Asp-Val-Tyr-		
U2	Val-Met-	-Ala-Ser-Asp-Phe-Tyr-		-Ser-Thr-
HR	Ala-Pro-Asp-Gln-	-Asp-Thr-Gly- -Arg-	-Val-Asn-Ser-Ala-Val-	

	80	85	90
TMV	Leu-Asp-Pro-Leu-Val-	Thr-Ala-Leu-Leu-Gly-	Ala-Phe-Asp-Thr-Arg-Asn-Arg-Ile-Ile-
D	-Ile-	-Thr-	
U2		-Asn-Ser-	
HR	Ile-Lys- -Tyr-Glu-	-Met-Lys-	

	95	100	105	110
TMV	Glu-Val-Glu-Asn-Gln-	Ala-Asn-Pro-	Thr-Thr-Ala-Glu-	Thr-Leu-Asp-Ala-Thr-Arg-Arg-
D		-Gln-Ser-		
U2	Glx-	-Asx-Asx-Glx-Ala-Asx-	-(Asx,Thr,Glx,Glx,Pro,Val,Ile)-	
HR	Gln-Thr-Glu-Glu-Gln-Ser-Arg-		-Ser-Ala-Ser-Gln-Val-Ala-Asn-Ala-Thr-Gln-	

	115	120	125	130
TMV	Val-Asp-Asp-Ala-Thr-	Val-Ala-Ile-Arg-	Ser-Ala-Ile-Asn-Asn-Leu-Ile-	Val-Glu-Leu-
D				-Val-Asn-
U2		-Ala-Ser-		-Ala-
HR		-Ser-Gln-	-Gln-Leu-	-Leu-

	135	140	145	150
TMV	Ile-Arg-Gly-Thr-Gly-	Ser-Tyr-Asn-Arg-	Ser-Ser-Phe-Glu-	Ser-Ser-Ser-Gly-Leu-Val-
D	Val-	-Leu- -Gln-Asn-Thr-	-Met-	
U2		-Met-Phe- -Ala-Gly-	-Thr-Ala-	
HR	Ser-Asx-His-Gly-	-Tyr-Met- -Arg- -Glu-	———Ala-Ile———	-Pro-

	155	158
TMV	Trp-Thr-Ser-Gly-Pro-	Ala-Thr
D	-Ala-	-Ser
U2	-Thr-Thr-	-Thr
HR	-Ala-	

[a] Adapted from Wittmann-Liebold and Wittmann 1967.

[b] TMV, common (vulgare) strain of tobacco mosaic virus; D, Dahlemense strain; U2, a mild strain; HR, Holmes' ribgrass strain.

Table 14. Sequence of Amino Acids in the Coat Proteins of Three Strains of Bacteriophage.[a]

```
     1                   5                  10                  15
fr   Ala-Ser-Asn-Phe-Glu-Glu-Phe-Val-Leu-Val-Asn-Asp-Gly-Gly-Thr-Gly-Asp-Val-
f2                    -Thr-Gln-                                       -Asn-
MS2                   -Thr-Gln-                 -Asp-Asn-

          20                  25                  30                  35
fr   Lys-Val-Ala-Pro-Ser-Asn-Phe-Ala-Asn-Gly-Val-Ala-Glu-Trp-Ile-Ser-Ser-Asn-
f2   Thr-
MS2  Thr-

                40                  45                  50
fr   Ser-Arg-Ser-Gln-Ala-Tyr-Lys-Val-Thr-Cys-Ser-Val-Arg-Gln-Ser-Ser-Ala-Asn-
f2                                                                   -Gln-
MS2                                                                  -Gln-

     55                  60                  65                  70
fr   Asn-Arg-Lys-Tyr-Thr-Val-Lys-Val-Glu-Val-Pro-Lys-Val-Ala-Thr-Gln-Val-Gln-
f2                       -Ile-                                       -Thr-Val-
MS2                      -Ile-                                       -Thr-Val-

           75                  80                  85                  90
fr   Gly-Gly-Val-Glu-Leu-Pro-Val-Ala-Ala-Trp-Arg-Ser-Tyr-Met-Asn-Met-Glu-Leu-
f2                                                     -Leu-    -Leu-
MS2                                                    -Leu-

                95                 100                 105
fr   Thr-Ile-Pro-Val-Phe-Ala-Thr-Asx-Asp-Asp-Cys-Ala-Leu-Ile-Val-Lys-Ala-Leu-
f2             -Ile-               -Asn-Ser-      -Glu-                  -Met-
MS2            -Ile-               -Asn-Ser-      -Glu-                  -Met-

          110                 115                 120                 125
fr   Gln-Gly-Thr-Phe-Lys-Thr-Gly-Ile-Ala-Pro-Asn-Thr-Ala-Ile-Ala-Ala-Asn-Ser-
f2        -Leu-Leu-   -Asp-    -Asn-Pro-Ile-Pro-Ser-
MS2       -Leu-Leu-   -Asp-    -Asn-Pro-Ile-Pro-Ser-

          129
fr   Gly-Ile-Tyr
f2
MS2
```

[a]Adapted from Wittmann-Liebold and Wittmann 1967; Min Jou et al. 1972.

a protein kinase, such as some of the RNA tumor viruses, do not have phosphate groups in their structural proteins, whereas the structural proteins of some viruses, such as simian virus 40, are all phosphoproteins despite the fact that the virus particles have no kinase (Tan and Sokol 1972). Therefore, the origin and function of these enzymes are unclear, although it has been suggested that production of phosphoproteins may be involved in the regulation of viral transcription.

Table 15. Amino Acid Sequences of The Coat Proteins of Bacteriophages ZJ-2 and fd.[a]

	1	5	10
ZJ-2	Ala-Glu-Gly-Asp-Asp-Pro-Ala-Lys-Ala-Ala		
fd			
	15		20
ZJ-2	Phe-Asp-Ser-Leu-Gln-Ala-Ser-Ala-Thr-Glu		
fd			
	25		30
ZJ-2	Tyr-Ile-Gly-Tyr-Ala-Trp-Ala-Met-Val-Val		
fd			
	35	40	
ZJ-2	Val-Ile-Val-Gly-Ala-Ala-Ile-Gly-Ile-Lys		
fd	-Thr-		
	45		50
ZJ-2	Leu-Phe-Lys-Lys-Phe-Thr-Ser-Lys-Ala-Ser		
fd			

[a]From Asbeck et al. 1969; Snell and Offord 1972.

B. Nucleic Acids

Nucleic acids are so named because they are acidic substances that were first isolated from the nuclei of cells.[1] It is now known that nucleic acids occur in both the nuclei and cytoplasm of all cells. The two major types of nucleic acid found in nature, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA), both occur in viruses. However, in contrast to bacteria and other organisms, no virus appears to contain both RNA and DNA. The type of nucleic acid present can be determined by qualitative tests for sugar and pyrimidine components since it is only with respect to these constituents that RNA and DNA differ in composition. The detection of deoxyribose and thymine indicate DNA, whereas the presence of ribose and uracil denote RNA (procedures for analysis of these substances are

[1]The properties of nucleic acids in general apply to viral nucleic acids. The interested student may wish to refer to such comprehensive reference works as *Progress in Nucleic Acid Research and Molecular Biology*, J. N. Davidson and W. E. Cohn, editors, New York: Academic Press (published annually since 1963); *Procedures in Nucleic Acid Research*, Vol. 1 and 2, G. L. Cantoni and D. R. Davies, editors, New York: Harper and Row (Vol. 1 in 1966 and Vol. 2 in 1971); *The Chemistry of Nucleosides and Nucleotides*, A. M. Michelson, New York: Academic Press (1963); *Genetic Elements—Properties and Function*, D. Shugar, editor, New York: Academic Press (1967); *Methods in Enzymology—Nucleic Acids*, L. Grossman and K. Moldave, editors, New York: Academic Press (Vol. XII, 1967; Vol. XII, Part B, 1968; Vol. XX, Part C, 1971; Vol. XXI, Part D, 1971; Vol. XXIX, Part E, 1974; Vol. XXX, Part F, 1974.

described by Ashwell 1957; Schneider 1957; Lin and Maes 1967; Burton 1968; Hatcher and Goldstein 1969).

The quantity of nucleic acid, while fairly constant within a given group of viruses, varies considerably among different viruses. The extremes are represented by 0.8 percent RNA in influenza viruses and about 56 percent DNA in coliphage lambda. Viral nucleic acids, like those from other sources, are elongated, threadlike molecules. Some of them are single stranded, some double stranded, and some are cyclic. The amount and type of nucleic acid found in some viruses are given in Table 16.

1. Preparation of Viral Nucleic Acids

Viral nucleic acid is deeply embedded in the protein matrix of the virus particle. Despite this sheltered location, the nucleic acid is accessible to some chemical reagents such as mustards, nitrous acid, formaldehyde, and smaller molecular species in general. Nevertheless, for many experiments, it is desirable to isolate the nucleic acid from the rest of the material. No single procedure has proved universally successful for this purpose. However, reagents noted for an ability to break secondary valence bonds, such as salt linkages and hydrogen and hydrophobic bonds, have been most effective in disaggregating virus particles with release of the nucleic acid.

To best study the properties and function of viral nucleic acids, it has become ever more important to isolate the intact nucleic acid, to the extent that this exists, from virus particles. Three main factors work against this objective: (1) mechanical shearing of the nucleic acid during isolation, (2) chemical degradation at the extreme pH values that favor removal of protein coats, and (3) enzymatic degradation.

Mechanical shearing is a problem primarily with large DNA molecules such as those found in phages. Violent mixing or even forceful pipeting of solutions of phage DNA are sufficient to rupture the molecules (Hershey et al. 1962) and breakage may also be accompanied by denaturation, that is, strand separation, under certain conditions of temperature and salt concentration (Hershey et al. 1963). Therefore, gentle stirring procedures are recommended in the isolation of nucleic acids.

The sensitivities of RNA and DNA to extreme pH values differ somewhat but the structures of both types of nucleic acid may be irreversibly altered at pH values below 3 or above 10. Below pH 3, depurination (cleavage of adenine and guanine) tends to occur with double-stranded molecules. RNA is subject to alkaline hydrolysis above pH 10, and DNA, while resistant to alkaline hydrolysis, may be denatured above pH 12. Consequently, most nucleic acid isolation procedures are performed at intermediate pH values.

Probably the greatest hazard to intact viral nucleic acid is attack by nuclease enzymes, that is, by ribonucleases and deoxyribonucleases.

Traces of nucleases can often be detected even in the most highly purified preparations of viruses; and while several of the methods for isolating nucleic acid include provision for protecting the product from nucleases, none of the procedures is entirely satisfactory in this regard. In principle, use of strong protein denaturants in removing the viral coat protein and releasing nucleic acid will also eliminate accompanying nucleases. In practice, however, unless the removal of denatured protein is complete, traces of nuclease will remain with the nucleic acid and subsequently become renatured and active (Ralph and Berquist 1967).

Since complete removal of denatured protein is difficult to ensure, one needs to combat nuclease activity in preparing viral nucleic acids by starting with the most highly purified virus obtainable, avoiding prolonged procedures, and by adding nuclease inhibitors. Two such inhibitors are the acid clay bentonite (Fraenkel-Conrat, et al. 1961; Singer and Fraenkel-Conrat, 1961) and diethyl pyrocarbonate (Solymosy et al. 1968; Bagi et al. 1970). Some disadvantages of these nuclease inhibitors are that bentonite adsorbs some RNA (Fraenkel-Conrat 1966) and diethyl pyrocarbonate under some conditions seriously inhibits the separation of viral protein and nucleic acid (Bagi et al. 1970).

In addition to the above factors, the salt concentration and nature of cations present can affect the isolation and stability of viral nucleic acids (Ralph and Berquist 1967). For example, at very low ionic strengths (10^{-4} M) strand separations occur in double-stranded nucleic acids and in double-stranded regions of single-stranded nucleic acids (all single-stranded nucleic acids have the tendency to form some double-stranded loops). Such denaturation usually makes nucleic acids more susceptible to degradation by nucleases. Cations such as Mg^{2+} may cause aggregation and loss of nucleic acids, especially of RNA, as do also salt solutions stronger than 1 M.

In summary, the various procedures for preparing intact undenatured viral nucleic acids are generally successful in proportion to their ability to effect a thorough denaturation of viral coat protein and separation of it from nucleic acid under conditions that avoid extremes of mechanical treatment, pH, and salt concentration and that minimize contact with nucleases. Some examples of procedures that apply these principles follow.

a. The Hot Salt Method

This procedure is a modification of the method of Cohen and Stanley (1942) (see Knight 1957; Reddi 1958; Lippincott 1961) that has proved useful in preparing nucleic acid from TMV and strains, although the nucleic acid isolated in this manner is not consistently so infectious as that obtained by detergent treatment or phenol extraction. [Infectivities comparable to the highest obtained by any procedure have been reported by Boedtker (1959) and by Lippincott (1961), using the hot salt method, but

Table 16. Approximate Content and Type of Nucleic Acid in Some Viruses.

Virus	Nucleic Acid per Particle, Daltons	Type of Nucleic Acid[a]	References
Algal N-1	38×10^6	ds-DNA	Adolph and Haselkorn 1971
Adenovirus-2	23×10^6	ds-DNA	Joklik and Smith 1972
Avian myeloblastosis	10×10^6	ss-RNA	Joklik and Smith 1972
Broad bean mottle	1×10^6	ss-RNA	Yamazaki et al. 1961
Brome mosaic	1×10^6	ss-RNA	Bockstahler and Kaesberg 1961
Coliphages f1, fd, M13	1×10^6	ss-c-DNA	Hoffmann-Berling et al. 1966
Coliphages f2, MS2, R17	1×10^6	ss-RNA	Hohn and Hohn 1970
Coliphages ØX174, S13	2×10^6	ss-c-DNA	Thomas and MacHattie 1967
Coliphage lambda	32×10^6	ds-DNA	Thomas and MacHattie 1967
Coliphages T2, T4, T6	130×10^6	ds-DNA	Thomas and MacHattie 1967
Cucumber 3 (and 4)	5×10^6	ss-RNA	Knight and Stanley 1941
Cytoplasmic polyhedrosis	13×10^6	ds-RNA	Kalmakoff et al. 1969
Foot-and-mouth disease	2×10^6	ss-RNA	Rueckert 1971

Fowlpox	200×10^6	ds-DNA	Hyde et al. 1967
Herpes simplex	100×10^6	ds-DNA	Joklik and Smith 1972
Influenza	4×10^6	ss-RNA	Compans and Choppin 1973
Mouse encephalitis	2×10^6	ss-RNA	Rueckert 1971
Newcastle disease	6×10^6	ss-RNA	Blair and Duesberg 1970
Poliomyelitis	2×10^6	ss-RNA	Schaffer and Schwerdt 1959
Polyoma	4×10^6	ds-c-DNA	Kass 1970
Potato X	2×10^6	ss-RNA	Reichmann 1959
Reo, Type 3	15×10^6	ds-RNA	Joklik 1970
Rous sarcoma	10×10^6	ss-RNA	Robinson and Duesberg 1967
Shope papilloma	5×10^6	ds-c-DNA	Kass and Knight 1965
Silkworm jaundice	22×10^6	ds-DNA	Bergold 1953; Bergold and Wellington 1954
Simian 40	4×10^6	ds-c-DNA	Yoshiike 1968
Tobacco mosaic	2×10^6	ss-RNA	Knight and Woody 1958
Tomato bushy stunt	2×10^6	ss-RNA	DeFremery and Knight 1955
Turnip yellow mosaic	2×10^6	ss-RNA	Markham 1959
Wound tumor	16×10^6	ds-RNA	Kalmakoff et al. 1969

[a]ds = double-stranded; ss = single-stranded; c = cyclic.

with temperatures between 90° and 98.5°. However, recoveries of RNA are substantially lower under these conditions and the risk of contamination with undegraded virus, higher.]

1. *Hot salt procedure for preparing RNA from TMV and similar viruses.* To 0.3 M NaCl in a water bath at 100°C is added enough virus in aqueous solution to give a final concentration of 10–15 mg/ml. After mixing, the mixture is held at 100° for 1 min and then removed to an ice bath. After chilling, the mixture is centrifuged at 5,000–10,000 *g* to remove coagulated protein. The water-clear solution of sodium nucleate can be freed of salt by dialysis against water in the cold or by precipitation two or three times with ice-cold 67 percent alcohol, redissolving the nucleate in water each time. (A final step of high-speed centrifugation may be used, if desired, to pellet traces of insoluble matter.) Yields of about 80 percent are usually obtained.

2. *Modified hot salt method for preparation of RNA from tobacco ringspot and turnip yellow mosaic viruses.* For success with tobacco ringspot and turnip yellow mosaic viruses, Kaper and Steere (1959a, 1959b) found it necessary to modify the hot salt procedure by reducing the virus concentration and heating time and increasing the salt concentration. Thus, to 2 ml of M NaCl in a boiling water bath is added 1 ml of virus (at 5–10 mg/ml in 0.01 M phosphate buffer at pH 7) and heating is continued for 35 sec with constant mixing. The mixture is cooled immediately in an ice bath and the coagulated protein is removed by centrifugation. The nucleic acid, in the supernatant fluid, can be purified by two precipitations with cold alcohol and high-speed centrifugation as above.

3. *Modified hot salt procedure for preparing RNA from influenza and Rous sarcoma viruses.* Only about 0.8 percent of influenza virus is RNA and it is difficult to extricate the nucleic acid from the great mass of protein, lipid, and carbohydrate present. A hot salt procedure reported to give very good yields was developed by Ada and Perry (1954). The method has also been used to extract RNA from Rous sarcoma virus (Bather 1957).

The purified, frozen-dried (lyophilized) virus is defatted by extracting twice at room temperature with chloroform-methanol (2:1, v/v) followed by one extraction with *n*-butanol and two washes with ethyl ether. The RNA is obtained by extracting the defatted virus one to three times at 100° with 10 percent (w/v) NaCl, using 20-min extraction periods. The RNA can be freed of salt as above, with alcohol precipitation probably preferable. So far, the nucleic acid thus obtained from influenza virus has proved noninfectious.

A hot salt method has also been used to prepare DNA from phage ØX174 (Guthrie and Sinsheimer 1960; Sekiguchi et al. 1960).

b. Detergent Method

The following procedure, adapted from the method of Fraenkel-Conrat et al. (1957), gives good yields of RNA from TMV and related viruses. The RNA is infectious (Fraenkel-Conrat 1956) and reconstitutes well with pro-

tein obtained by acetic acid degradation of the virus (see section on Reconstitution of Viruses).

1. *Detergent procedure for isolating RNA from TMV and similar viruses.* Virus at 20 mg/ml in water is heated to 55° in a water bath, adjusted to pH 8.8 with dilute NaOH, and mixed with an equal volume of 2 percent sodium dodecyl sulfate (commercial preparations such as Duponol C are satisfactory also) that has also been adjusted to pH 8.8 at 55°. The mixture is allowed to remain in the water bath at 55° for 5 min during which the solution loses its characteristic opalescence, owing to degradation of the virus. After 5 min (greater or less time may be required for different strains of TMV) the mixture is rapidly cooled to room temperature (about 23°C) and 0.5 vol of saturated ammonium sulfate is added. After about 10 min the precipitated protein is removed by centrifugation at 5,000–10,000 *g* and the clear supernatant fluid is stored at 4° overnight. The RNA precipitates out under these conditions, and the precipitate is packed by centrifugation, redissolved in a small volume of water, and reprecipitated by adding 2 vol of cold alcohol. The alcohol precipitation may be repeated once or twice more and traces of insoluble material may be removed from the final solution of RNA by centrifuging at about 100,000 *g* for 2 hr with refrigeration. Yields of 60–90 percent are obtained.

2. *Modified detergent procedure for isolation of DNA from polyoma virus (Smith el al. 1960).* Polyoma virus was isolated from clarified extracts of a mouse embryo tissue culture by differential and density gradient centrifugation. Equal volumes of virus solution and 10 percent sodium dodecyl sulfate at pH 7 are mixed and heated at 65° for 2 hr. After adding enough ammonium acetate to give a final concentration of 0.1 M, the DNA is precipitated by adding 2 vol of ethanol. The precipitate is dissolved in 0.1 M ammonium acetate and reprecipitated by adding alcohol as before. This dissolving and precipitating procedure is repeated once more.

A modified detergent method has also been used in the isolation of DNA from Shope papilloma virus (Watson and Littlefield 1960).

c. Combined Detergent and Hot Salt Method

Some viruses from which low yields of nucleic acid are obtained by either the hot salt or detergent methods alone will give reasonable amounts of nucleic acid by a combined procedure (Dorner and Knight 1953). In this method, 1 vol of 10 percent Duponol C solution (or sodium dodecyl sulfate) is added to 4 vol of aqueous virus at about 10 mg/ml. The mixture is heated in a boiling water bath for 4 min and then cooled in an ice bath. Most of the free detergent is removed by dialysis and then enough 5 N NaCl is added to make the final concentration 1 N with respect to NaCl. This mixture is heated for 3 min at 100°, chilled in an ice bath, and the coagulated protein is removed by centrifugation. Salt is removed by dialysis and the preparation is concentrated by directing a stream of air at the dialysis bag (pervapora-

tion). The concentrate of nucleate can be clarified by centrifugation, or, if desired, the nucleate can be precipitated from the concentrate by addition of 2 vol of cold ethanol.

d. The Phenol Method

The phenol extraction method (Westphal et al. 1952) is perhaps the most generally useful procedure for obtaining nucleic acid from a wide variety of viruses (as well as from tissues). In operation, two layers—a phenolic and an aqueous layer—are obtained and protein is extracted into the phenolic layer (and some in the interface) while nucleic acid (and polysaccharide, if present) goes into the aqueous layer.

Phenol extraction was first used to prepare viral nucleic acid by Schuster et al. (1956), and very soon it was shown (Gierer and Schramm 1956a, 1956b) that RNA thus obtained from TMV is infectious. The initial procedure does not work satisfactorily on all viruses, but in several cases modifications have been developed that have successfully extended the usefulness of the technique. A convenient adaptation of the method for the preparation of RNA from TMV and strains is presented here together with some modifications extending the usefulness of the method.

1. Phenol procedure for preparing RNA from TMV and similar viruses. To the virus solution in 0.02 M phosphate buffer at pH 7 and at a virus concentration of 20–25 mg/ml is added an equal volume of water-saturated phenol. (This is about 80 percent phenol, and it is easily prepared by taking a fresh bottle of commercial reagent grade crystalline phenol and almost filling the bottle with distilled water. The mixture is liquefied by placing in a warm water bath and stirring occasionally. Two layers will be apparent: a large lower layer consisting of the water-saturated phenol and a small upper layer of excess water. If stored at about 4° in the dark glass bottle normally commercially available, the preparation keeps for weeks, and portions of the lower layer are used in the preparation of nucleic acid. Some workers redistill their phenol, add metal-chelating agents such as sodium versenate, and so on, but the author has not found these refinements to be generally necessary.)

The mixture of virus and phenol is stirred on a magnetic stirrer for 10–15 min at room temperature (about 23°C), after which the mixture is separated into two layers by centrifuging at 5,000–10,000 *g* for about 2 min. (The original procedure was carried out at low temperature, and 4° is still used in some cases; in other instances it has been found necessary to use temperatures as high as 50°–60°.) The aqueous (top) layer is drawn off, and about one-tenth its volume of water-saturated phenol is added, and the mixture is stirred again for 3–4 min followed by centrifugation. The aqueous layer is extracted once more with a tenth volume of phenol and then twice with equal volumes of ether (to remove the small amount of phenol which dissolves in the aqueous phase). Residual ether is removed from the aque-

ous nucleate by two to three precipitations of the RNA with ethanol, which is accomplished by chilling the nucleate solution and adding 2 vol of ice-cold ethanol. If difficulty in precipitating the material is experienced, a drop or two of 3 M sodium acetate at pH 5 can be added. The final precipitate, pelleted by centrifugation, is dissolved in a small volume of distilled water and centrifuged at about 100,000 *g* for 2 hr. The nucleic acid is not sedimentable under these conditions but a trace of insoluble material is usually removed as a tiny pellet. Yields of the order of 80 percent are commonly obtained.

Conditions similar to those described above (except that usually temperatures around 4° have been employed without evidence that such low temperatures are necessary) have been used successfully to prepare RNA from partially purified poliovirus (Alexander et al. 1958); from potato virus X (Bawden and Kleczkowski 1959); from tobacco rattle virus (Harrison and Nixon 1959b); from cucumber mosaic virus (Schlegel 1960b); from an RNA-containing insect virus, Smithia virus pudibundae (Krieg 1959); and others. The method has also been used to extract DNA from T2, T4, and ØX174 phages (Davison et al. 1961; Rubenstein et al. 1961; Guthrie and Sinsheimer 1960).

e. Phenol-Detergent Method

In some cases it has been found beneficial to make the phenol extraction after the virus structure has been opened up by a detergent such as sodium dodecyl sulfate. Rushizky and Knight (1959) used such a technique to obtain infectious RNA from tomato bushy stunt virus, Huppert and Rebeyrotte (1960) to extract DNA transforming principle from bacteria, Wahl et al. (1960) to prepare ØX174-DNA, and Bachrach (1960) to obtain infectious RNA from foot-and-mouth disease virus. Bachrach's method is given here.

> Virus concentrates are diluted six times in 0.02 M phosphate buffer at pH 7.6 which contains 0.01 percent sodium ethylenediaminetetraacetate (EDTA, or "Versene") and 0.1 percent sodium dodecyl sulfate. The diluted virus is twice extracted at 4° with water-saturated phenol containing 0.01 percent EDTA. Phenol is removed from the final aqueous phase by several extractions with ether. The ether is removed by a stream of nitrogen.

In the standard phenol procedure used for preparation of RNA from TMV and similar viruses, the extraction is now made at room temperature, which is about 20° warmer than used in the original procedure. Even this temperature, however, is not sufficient for some viruses, and it has been necessary to go to about 50°, for example, to extract the RNA from equine encephalomyelitis virus (Wecker 1959).

Another important modification of the phenol method involves the

addition of bentonite as an adsorbent for nucleases during the phenol extraction (Fraenkel-Conrat et al. 1961) The infectivity of RNA obtained from TMV by the phenol-bentonite procedure is stabler upon incubation in salt solutions than most preparations made without bentonite (Singer and Fraenkel-Conrat 1961).

f. Guanidine Hydrochloride Method

Bawden and Kleczkowski (1959) reported the preparation of infectious RNA from potato virus X by phenol extraction, but the reproducibility of the results was not good. Therefore Reichmann and Stace-Smith (1959) investigated other procedures and devised a method based on treatment with guanidine that gave consistently 70–90 percent yields of infectious RNA. Their method is as follows:

To virus solution at 5–10 mg/ml is slowly added a sufficient volume of concentrated, recrystallized guanidine hydrochloride and ethylenediaminetetraacetate at pH 8.4 to make a final concentration of 2.5 M in guanidine and 0.005 M in EDTA. After 1 hr the RNA, which is insoluble, and the protein, which is soluble, in this mixture are separated by centrifugation at about 4,500 *g*. The RNA pellet is washed twice with 2.5 M guanidine-EDTA solution and then dissolved in a small volume of water. Further purification of the RNA is accomplished with 2 vol of cold ethanol, a 90-min centrifugation at 75,000 *g*, and dialysis overnight against distilled water.

g. Alkaline Method

As discussed earlier, extremes of pH are generally to be avoided in the preparation of nucleic acids. However, DNA is fairly resistant to alkaline media up to pH 12, and this fact was taken advantage of in isolating the nucleic acid of a nuclear polyhedrosis virus of the silkworm (*Bombyx mori*) (Onodera et al. 1965).

Freshly prepared virus particles are suspended in a small amount of 0.1 M Na_2CO_3–$NaHCO_3$ buffer (pH 10) 0.1 M in sodium citrate. This is dialyzed against a large volume of the same buffer-citrate mixture at 4° for two days. The dialyzed material is centrifuged at 40,000 *g* for 30 min to remove insoluble material. Solid ammonium sulfate is added to the supernatant fluid to a concentration of about 25 percent (wt/vol). The resulting precipitate of protein is removed by centrifugation and the supernatant containing the nucleic acid is dialyzed at 4° against 0.15 M NaCl–0.15 M sodium citrate. The DNA can be further purified by precipitation with 2–3 vol of cold ethanol or by passage through a column of methylated albumin.

In general, it may be stated that isolated viral nucleic acids are stabler than at first supposed. The primary cause of the instability is apparently traces of nucleases, and if these are absent, the nucleic acids maintain their integrity on storage and withstand temperatures considerably above room

temperature. In short, nucleic acids are not intrinsically chemically unstable, nor are they particularly thermolabile.

2. *Analysis of Viral Nucleic Acids*

Nucleic acids may be considered to be polynucleotides, that is, chain-like molecules in which the links are nucleotides. This is illustrated in Figure 17, which depicts the essential features while showing only a very small segment of nucleic acid.

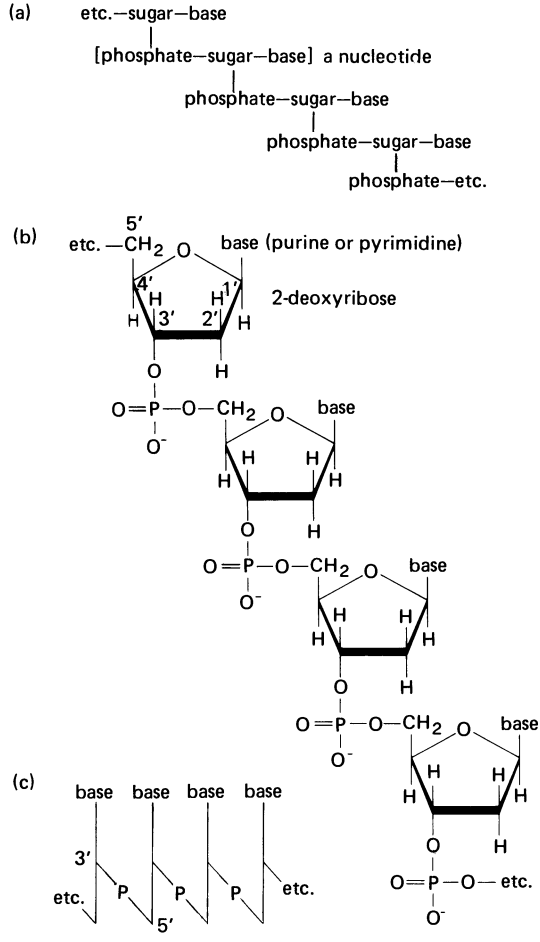
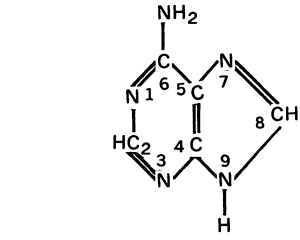


Fig. 17. Composition of a small segment of nucleic acid indicating the order of arrangement of the three major components (sugar, base, and phosphate). *a*. General scheme of sugar-phosphate backbone structure. *b*. Chemical structure of sugar-phosphate backbone of DNA showing numbering of atoms in the sugar (deoxyribose) and phosphodiester linkages between nucleotides. *c*. An abbreviated way to indicate oligonucleotides. (Adapted from Knight 1974.)

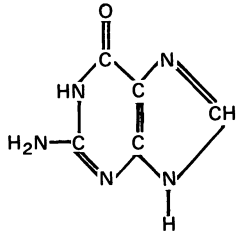
Nucelotides are named according to the purine or pyrimidine base they contain. In the case of DNA, which contains deoxyribose rather than ribose as in RNA, this is indicated in the naming of the nucleotides by appending the prefix deoxy. Thus, for RNA, the nucleotides are adenylic acid, guanylic acid, cytidylic acid, and uridylic acid; for DNA they are deoxyadenylic acid, deoxyguanylic acid, deoxycytidylic acid, and thymidylic acid (the deoxy prefix is not necessary for the thymine-containing nucleotide since there is no natural counterpart in the ribonucleic acid series).

As indicated in Figure 17, all nucleotides are built up from three simpler components: phosphate, sugar, and a purine or pyrimidine base. Nucleosides are made from sugar and a purine or pyrimidine base. Nucleosides are thus chemically closely related to nucleotides, and removal of phosphoric acid (by hydrolysis) from a nucleotide yields a nucleoside. Conversely, nucleotides can be viewed as nucleoside phosphates. The common ribonucleosides are adenosine, guanosine, cytidine, and uridine. The comparable deoxyribonucleosides are deoxyadenosine and so on, except that deoxy is commonly omitted from the name of the nucleoside consisting of deoxyribose and thymine (thymidine) since thymidine is characteristic of DNA only.

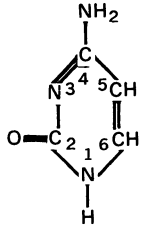
The purine bases commonly found in nucleic acids are adenine and guanine and the pyrimidines are cytosine, uracil, and thymine, the latter occurring only in DNA. In the DNA of certain phages, 5-hydroxymethylcytosine or 5-hydroxymethyluracil is found in place of cytosine. Formulas for some of these bases are as follows:



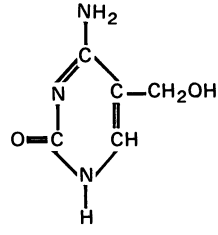
Adenine
(6-aminopurine)



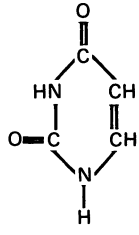
Guanine
(2-amino-6-oxypurine)



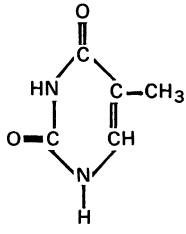
Cytosine
(2-oxy-4-amino-pyrimidine)



5-Hydroxymethyl-cytosine

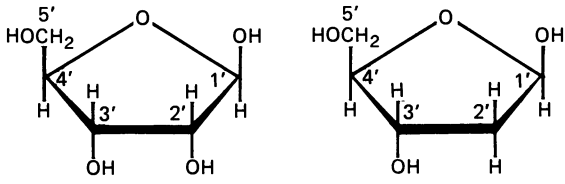


Uracil
(2,4-dioxypyrimidine)



Thymine
(5-methyluracil)

The sugar components of RNA and DNA are D-ribose and 2-deoxy-D-ribose, respectively. These sugars in the β configuration (see the following formula) are attached to purines and pyrimidines in nucleic acids (and also in nucleotides and nucleosides). Both sugars are found in nucleic acids (and in nucleotides) in the furanose ring form (oxygen ring between carbons 1′ and 4′), whereas the free sugars occur mainly in the pyranose form (oxygen ring between carbons 1′ and 5′). Structural and abbreviated formulas may be written for these sugars as follows:



It will be noted in the formulas for nucleic acid components that regular numbers were used to denote positions in the purine and pyrimidine rings, whereas prime numbers were used to indicate positions in the sugars. This convention applies to nucleosides, nucleotides, and nucleic acids in order to make the distinction between derivatives involving the sugar and those affecting the bases.

In nucleic acids, including those of viruses, the nucleotides are uniformly linked through 3′ → 5′ phosphate diester bonds between the sugar moieties as illustrated in the bit of DNA shown in Figure 17. This means that all DNA molecules share a common deoxyribose-phosphate structure; similarly all species of RNA have a common ribose-phosphate backbone. Consequently, aside from differences in molecular size, the biological specificity of nucleic acids depends entirely on the purine and pyrimidine bases, or, more precisely, on the sequence in which these bases occur along the sugar-phosphate chain.

An important step in characterizing nucleic acids is to determine the proportions of purines and pyrimidines present. This is commonly done by hydrolyzing the nucleic acid and separating the resultant components of the hydrolysate by either of three methods: paper chromatography or thin layer chromatography, paper electrophoresis, or column chromatography. Concentrations of discrete fractions separated by one of these methods are usually determined by ultraviolet spectrophotometry. Examples of some of the procedures are given below.

a. Determination of the Base Ratios in RNA by Acid Hydrolysis and Paper Chromatography

Numerous studies have been made on the hydrolysis of nucleic acids and the separation of nucleic acid constituents (see, for example, Fink and

Adams 1966; Lin and Maes 1967). An early and still useful procedure for determining base ratios in RNA is that of Smith and Markham (1950). Mild acid hydrolysis is used, which releases the purines in the form of free bases and the pyrimidines in the form of their nucleotides.

Viral RNA, isolated by one of the methods described in the previous section, is hydrolyzed at a concentration of 10 mg RNA/ml in 1 N HCl for 1 hr at 100° (boiling water bath). Twenty μl (equivalent to 200 μg of RNA) of hydrolysate is applied to Whatman No. 1 filter paper with a micropipette in such a way as to give a rectangular spot about 0.5 × 5 cm. Separation of the components is effected by either ascending or descending chromatography in 70 percent tert. butanol-water, 0.8 N with respect to hydrochloric acid (70 ml tert. butanol, 13 ml constant boiling HCl, and 17 ml H_2O). After chromatography, the papers are air-dried at room temperature, and the spots are located by examination with an ultraviolet light and marked with a pencil. Starting at the point of application and going in the direction of the solvent movement, the spots will be found in the following order: guanine, adenine, cytidylic acid, and uridylic acid. The sample spots are cut out, as well as paper blanks of approximately the same size next to the sample spots. Each paper cutout is placed in a test tube together with 5 ml of 0.1 N HCl and eluted by standing at room temperature overnight or by shaking for 2 hr. The absorption of each solution is read in a spectrophotometer at the wavelength of maximum (or near maximum) absorption for the compound in question. The amount of each component can be calculated by use of the proper extinction coefficients such as those given by Sober (1970). Some data of this sort are given in Table 17 for the compounds obtained from RNA as above, and for the compounds obtained by alkaline hydrolysis of RNA or acid hydrolysis of DNA.

During hydrolysis in 1 N HCl, about 5 percent of the pyrimidine

Table 17. Ultraviolet Absorption Data for Some Nucleic Acid Constituents Near Wavelengths of Maximum Absorption.[a]

Substance	pH	Wavelength, nm	Molar Extinction Coefficient, × 10^{-3}
Adenine	1	262.5	13.2
Guanine	1	248	11.4
Cytosine	1	276	10.0
5-Hydroxymethylcytosine	1	279	9.7
Thymine	4	265	7.9
Uracil	0	260	7.8
Adenylic acid	1	257	15.0
Guanylic acid	1	257	12.2
Cytidylic acid	2	279	13.0
Uridylic acid	1	262	10.0

[a]Compiled from Sober 1970.

nucleotides are hydrolyzed to nucleosides. Hence for more accurate values using this method, the cytidylic and uridylic acid figures can be corrected upward by 5 percent (Markham and Smith 1951) and the adenine value downward by the same amount (cytidine arising from partial hydrolysis of cytidylic acid migrates to the same area as adenine in tert. butanol-HCl. Uridine occupies an area between cytidylic and uridylic acids and hence does not affect values for other components). A more precise correction can be made by actual determination of the amount of cytidine in the adenine spot. This is done by using observed absorption values at two wavelengths, standard absorption values, and applying simultaneous equations (Loring and Ploeser 1949). Assuming a similar conversion of uridylic acid to uridine permits complete correction and accounts very well for the nucleic acid components in terms of phosphorus recovery (deFremery and Knight 1955).

The hydrolysis in 1 N HCl is capable of releasing the nucleic acid components from whole virus as well as from isolated nucleic acid (Dorner and Knight 1953). Hence it is not necessary to isolate nucleic acid in order to determine the proportions of purines and pyrimidines present. However, this analysis is affected somewhat by the relative proportions of protein and nucleic acid, and gives most accurate results on the viruses containing 10 percent or more RNA.

b. Determination of the Base Ratios in RNA by Alkaline Hydrolysis and Paper Electrophoresis

Another convenient method for determining the base composition of RNA is by hydrolizing the nucleic acid in dilute alkali at low temperature, separating the resulting four nucleotides by paper electrophoresis, and determining the quantity of each nucleotide by spectrophotometry on the material eluted from the paper (see Smith 1955; Crestfield and Allen 1955a, 1955b). A useful procedure is as follows.

Two mg of RNA is hydrolyzed in 0.1 ml of 0.4 N NaOH at 37° for 24 hr. Ten μl aliquots of this hydrolyzate is applied to buffer-moistened Whatman 3 MM paper in an approximately 2-cm streak. Any of a number of types of electrophoresis apparatus may be used (See Smith 1955; Crestfield and Allen 1955a; Rushizky and Knight 1960b). The buffer used is 0.05 M formate at pH 3.5 (prepared by adding 6.4 g ammonium formate and 10.3 g formic acid, 88–90 percent, to 6 liters of water). Electrophoresis is performed at a voltage gradient of 6 v/cm for about 15 hr. (With proper cooling, a higher voltage can be used and the separation accelerated.) After drying the paper in air, the nucleotides can be located by examination with an ultraviolet light, marked, cut out (with appropriate blanks), eluted in 5-ml portions of 0.01 N HCl, and measured in a spectrophotometer. At pH 3.5 the nucleotides are found in the following order, starting from the cathode side of the paper: cytidylic acid, adenylic acid, guanylic acid, and uridylic acid.

c. Determination of the Base Ratios in DNA by Acid Hydrolysis and Paper Chromatography

Analyses of DNA are based on methods of acid hydrolysis that release the purines and pyrimidines as free bases. Either 70 percent perchloric acid or 88 percent (or 98 percent) formic acid are usually employed. Wyatt (1955) suggests the use of formic acid for best recoveries of the various bases (including the somewhat labile 5-hydroxymethylcytosine), but hydrolyses must be made in sealed bomb (thick-walled) tubes and the pressure from decomposition of formic acid is conveniently released, after hydrolysis and cooling, by heating a small area at the top of the tube until a little hole blows open. The tube may then be safely and fully opened. If it is desired to get base analyses on whole virus without isolation of the nucleic acid, then 70 percent perchloric acid is recommended. The following is a possible procedure based on these observations.

DNA is placed in a pyrex glass bomb tube and enough 88 percent formic acid is added to give a concentration of 2 mg DNA/ml formic acid. The tube is sealed and heated at 175° for 30 min. After cooling, pressure is released as described above; the tube is opened and the hydrolysate is evaporated to dryness in vacuo. The residue is taken up in a small volume of N HCl to give a concentration equivalent to 10–20 mg/ml of the original DNA. Twenty μl of hydrolysate is placed on Whatman No. 1 paper and chromatographed in isopropanol-HCl-water (170 ml isopropanol, 41 ml concentrated HCl, 39 ml H_2O). The migration of the bases in increasing distance from the origin is in the order guanine, adenine, cytosine, and thymine. In cases where 5-hydroxymethylcytosine is present instead of cytosine, it will be found in the cytosine position. Location of the spots and elution and spectrophotometry are carried out as above.

d. Determination of the Nucleotide Ratios in ^{32}P-Labeled RNA by Alkaline Hydrolysis and Column Chromatography

An example of analysis of a phage RNA using column chromatography can be drawn from the studies of coliphage β (Nonoyama and Ikeda 1964). Coliphage β was grown in *E. coli* K12 bacteria in the presence of ^{32}P so that this was incorporated in the phage RNA. Radioactive phage RNA extracted from the virus by the phenol procedure was mixed with carrier yeast RNA (use of carrier enables analysis of minute amounts of viral nucleic acid) and the mixture was hydrolyzed to nucleotides by treatment with 0.3 N NaOH for 18 hr at 37°. The hydrolysate was neutralized with 0.3 N HCl and loaded on a Dowex column (formate type, 1 × 2). The nucleotides were separately eluted with a gradient of formic acid (0–4 N) and the radioactivities of the issuing fractions were measured in an automatic gas-flow counter. From these data and the assumption of equivalent labeling of the different nucleotides, the composition of the RNA could be calculated.

e. Determination of Base Ratios in DNA from Buoyant Density and Thermal Denaturation Values

By examination of many different samples it has been determined that the buoyant density of DNA in cesium chloride is directly proportional to its guanine plus cytosine content (the buoyant density of a substance is equivalent to the density of solution at the equilibrium position to which the substance sediments in a density gradient). Deviations from this linear relationship occur only if the purine or pyrimidine bases are substituted, that is, if the DNA contains bases other than adenine, thymine, guanine, or cytosine. Such cases are rare. Hence, by density-gradient centrifugation of viral DNA, together with a marker DNA of known density, data are obtained enabling the calculation of the viral DNA. These data are obtained by use of a centrifuge equipped to record the positions of the sedimenting species from their ultraviolet absorbancies. A detailed description of the technique and an illustrative calculation of density are given by Mandel et al. (1968). From a curve representing the best fit of measurements made on 51 DNA samples, Schildkraut et al. (1962) developed the relation

$$(\mathrm{GC}) = \frac{\rho - 1.660\ \mathrm{g/ml}}{0.098}$$

where (GC) is the mole fraction of guanine plus cytosine and ρ is the buoyant density of the DNA in CsCl.

Similarly, a linear relationship exists between the molar percentage of guanine plus cytosine in DNA and the denaturation or "melting" temperature (T_m) of the nucleic acid. From observations on 41 samples of DNA, Marmur and Doty (1962) developed the relation: (GC) = (T_m-69.3) 2.44, where (GC) is the mole percentage (note that mole percentage = mole fraction × 100) and T_m is in degrees centigrade in a solvent containing 0.2M Na^+. The absorbance of the DNA solution at 260 nm is measured as a function of temperature, and T_m is taken at the midpoint of the increase in absorbance (hyperchromic rise). Details for the performance of such measurements are given by Mandel and Marmur (1968).

The errors associated with both the density and thermal denaturation procedures appear to be small and both procedures can be performed with microgram amounts of DNA. A comparison of some results obtained by these procedures and those obtained by chemical analysis are illustrated in Table 18.

An example of the use of such data as those in Table 18 is as follows. Using either the buoyant density or thermal denaturation temperature value for guanine plus cytosine listed for herpesvirus DNA in Table 18, and applying the molar equivalence rule that applies to the bases of double-stranded DNA (see Sec. f), one can readily calculate that the molar percentages of bases in this DNA are 16 percent adenine, 16 percent thymine, 34 percent guanine, and 34 percent cytosine.

Table 18. Guanine Plus Cytosine Content of Some Viral DNAs.

Virus	Molar Percentages of Guanine plus Cytosine			References[a]
	From Chemical Analysis	From Buoyant Density	From Thermal Denaturation Temp.	
Coliphage T3	50	53	49	1,2
Coliphage T7	48	51	48	1,2
Coliphage lambda	49	51	47	1,2
Adenovirus-2	58	57	57	3
Herpes	74	68	68	4,5
Shope papilloma	48	50	49	6

[a](1) Schildkraut et al. 1962. (2) Marmur and Doty 1962; (3) Piña and Green 1965; (4) Ben-Porat and Kaplan 1962; (5) Russell and Crawford 1963; (6) Watson and Littlefield 1960.

f. Proportions of Nucleotides in Some Viral Nucleic Acids

There are different ways of expressing the results of the base analyses made on nucleic acids. The commonest are (1) an arbitrary basis such as (a) moles of base per total of 4 moles, or (b) any one of the bases is set equal to 1 (or 10) and the values of the other bases are calculated accordingly; (2) moles percent, that is, moles base per 100 moles total bases; (3) moles base per mole phosphorus. Method 3 is probably to be preferred since it permits a ready evaluation of the recovery of the bases (there should be a total of 1 mole of bases per mole of phosphorus). However, this requires phosphorus analyses to be made, and sometimes there is not enough sample to make the desired replicate base determinations and phosphorus analyses too. Finally, since there is one base per nucleotide, results may obviously be expressed interchangeably in terms of moles base or moles of nucleotide. The compositions of some viral nucleic acids are summarized in Table 19 in terms of mole percent of nucleotides.

Several points about the compositions of viral nucleic acids as listed in Table 19 may be noted in passing. Nucleic acids of plant and mammalian tissues often contain small amounts of 5-methyldeoxycytidine (Hall 1971). However, such methylation rarely appears among viral nucleic acids. Two exceptions are noted in Table 19: 5-hydroxymethylcytosine found in the T-even coliphages and 5-hydroxymethyluracil observed in the DNA of *B. subtilis* phage SP8. In fact, on the basis of current information unusual purines or pyrimidines are quite uncommon in viral nucleic acids. One interesting variation is the occurrence of deoxyuridylic acid rather than thymidylic acid in the DNA of *B. subtilis* phage PBS2.

Inspection of the molar proportions of nucleotides for different viruses (Table 19) indicates considerable variation among viruses in this regard. Two examples, the DNAs of coliphage T3 and of *Salmonella* phage P22, appear to have equimolar proportions of all four constituent nucleotides. This is fortuitous and does not mean that the four nucleotides occur repeti-

tively in tandem (old tetranucleotide hypothesis). In contrast, the nucleic acids of potato virus X and of white clover mosaic virus have unusually large proportions of adenine; those of herpes simplex and pseudorabies, similarly big proportions of guanine; those of turnip yellow mosaic and wild cucumber mosaic viruses, extraordinary amounts of cytosine; and coliphages of the f1 group and influenza virus have lopsided proportions of thymine and uracil, respectively. The nucleic acids of most of the other viruses listed in Table 19 have undistinctive compositions.

While there are distinctive differences in the compositions of most of the nucleic acids of different viruses, such distinctions are seldom demonstrable with strains of the same virus. For example, the composition given for TMV suffices for its various strains, and one composition can be given for five strains of influenza A virus, one for the T-even coliphages, one for coliphages of the f1 series, one for three types of poliovirus, and so on. It should be remembered, however, that the present methods of analysis have an accuracy of about ± 3 percent, which, for example, is equivalent to about ± 50 nucleotides for any of the four nucleotides of TMV-RNA and of course proportionately larger for the bigger nucleic acids. This analytical situation should be viewed in the context that a difference in one nucleotide may be biologically significant.

The nucleic acids of viruses containing either double-stranded DNA or double-stranded RNA exhibit a molar equivalence of bases first noted in some DNAs by Chargaff and associates (1955):

$$\frac{\text{Adenine}}{\text{Thymine (or uracil)}} = \frac{\text{Guanine}}{\text{Cytosine (or 5-HMC)}} = \frac{\text{Purines}}{\text{Pyrimidines}} = 1$$

These regularities have definite implications concerning the structure of DNA (and of RNA) and were instrumental in the development of the Watson-Crick (1953a) double helix model.

g. Polynucleotide End Groups and Other Structural Features

In the case of proteins, primary structure analysis involves determination of numbers of chains in the protein molecule and the sequence of amino acid residues in the chain or chains. This concept, somewhat modified, can be carried over into nucleic acid structure, and, in the case of viruses, involves determination of the number of polynucleotide chains per virus particle and the sequence of nucleotides in the chain or chains.

With respect to these analyses, two points can be stated at the outset: (1) It is very common for viruses to have a single molecule or chain of nucleic acid per virus particle, but there are several instances, particularly in the case of viruses with double-stranded RNA in which the nucleic acid is segmented, that is, occurs in 10 to 15 discrete pieces per particle. (2) Sequencing of nucleic acids at present is more difficult than sequencing

Table 19. Amount of Nucleic Acid and Nucleotide Ratios of Some Viral Nucleic Acids.[a]

Virus	Type NA[b]	Daltons NA per particle, $\times 10^{-6}$	Approximate Moles Nucleotide per 100 moles					
			Ap[c] or dAp	Gp or dGp	Cp or dCp	Up or dUp[d]	Tp	5 HMdCp[e] or 5 HMdUp
Adenovirus 2	DNA	23	21	29	29		21	
Avian myeloblastosis	RNA	10	25	29	23	23		
B. subtilis PBS2	DNA	190	36	14	14	36[d]		
B. subtilis SP8	DNA	120	28	22	22			28[e]
Broadbean mottle	RNA	1	27	25	19	29		
Brome mosaic	RNA	1	27	28	21	24		
Coliphages f1, fd, M13	DNA	1	24	20	21			35
Coliphages f2, fr, M12, MS2, R17, β	RNA	1	23	26	26	25		
Coliphages ØX174, ØR, S13	DNA	2	24	25	19		32	
Coliphage Qβ	RNA	1	22	24	25	29		
Coliphages T2, T4, T6	DNA	120	33	17			33	17[e]
Coliphage T3	DNA	—[f]	25	25	25		25	
Coliphage lambda	DNA	30	26	24	24		26	
Coliphage T5	DNA	77	30	20	20		30	
Coliphage T7	DNA	24	26	24	24		26	
Cucumber 4	RNA	2	26	26	19	29		
Cytoplasmic polyhedrosis	RNA	13	29	21	21	29		
Foot-and-mouth disease	RNA	2	26	24	28	22		
Fowlpox	DNA	200	32	18	18		32	
Herpes simplex	DNA	81	16	34	34		16	
Influenza	RNA	4	23	20	24	33		
Mouse encephalitis	RNA	2	25	24	24	27		

Newcastle disease	RNA	6	24	24	23	29	
Poliomyelitis	RNA	2	29	24	22	25	
Polyoma	DNA	4	26	24	24		26
Potato X	RNA	2	32	22	24	22	
Pseudorabies	DNA	55	14	36	36		14
Reo Type 3	RNA	15	28	22	22	28	
Rous sarcoma	RNA	10	25	28	24	23	
Salmonella P22	DNA	28	25	25	25		25
Shope papilloma	DNA	5	26	24	24		26
Silkworm jaundice	DNA	22	30	20	20		30
Simian 40 (SV40)	DNA	5	26	24	24		26
Sindbis	RNA	—	29	26	25	20	
Tipula iridescent	DNA	156	34	16	16		34
Tobacco mosaic	RNA	2	28	24	22	28	
Tobacco necrosis	RNA	2	28	26	22	26	
Tobacco necrosis satellite	RNA	0.4	28	25	22	25	
Tobacco ringspot	RNA	2	24	25	23	28	
Tomato bushy stunt	RNA	2	26	28	21	26	
Turnip yellow mosaic	RNA	2	23	17	38	22	
Vaccinia	DNA	160	30	20	20		30
White clover mosaic	RNA	2	33	16	23	28	
Wild cucumber mosaic	RNA	3	18	16	40	26	
Wound tumor	RNA	16	31	19	19	31	

[a]Adapted from Knight 1974.
[b]NA is nucleic acid.
[c]Ap is adenylic acid, dAP is deoxyadenylic acid, and so on (see text for naming of nucleotides).
[d]*B. subtilis* phage PBS 2 is unusual in that it has deoxyuridylic acid in place of thymidylic acid, which is characteristic of DNA.
[e]5HMdCp is 5-hydroxymethyldeoxyytidylic acid which is found in coliphages T2, T4, and T6; and 5HMdUp is 5-hydroxymethyldeoxyuridylic acid found in *B. subtilis* phage SP8.
[f]–is unreported.

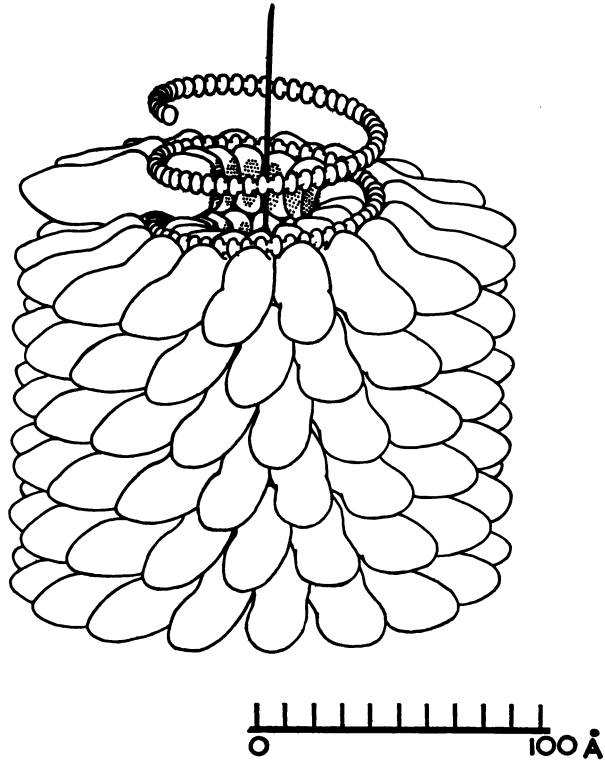


Fig. 18. Drawing of a segment of the tobacco mosaic virus particle with the protein subunits removed from the top two turns of the protein helix but maintaining the configuration of the RNA strand as it would be if the protein were there. The fit of the RNA in a helical groove of the protein subunits is indicated and individual nucleotides are denoted on the RNA strand as bead-like objects. It will be noted that there are about three nucleotides per protein subunit. (From Klug and Caspar 1960.)

proteins and, while extensive progress has been made, such sequencing is far behind that of proteins.

Progress in the chemical characterization of viral nucleic acids has been most pronounced with certain plant and phage nucleic acids. Therefore, these nucleic acids will be mainly used to illustrate some techniques and principles applicable to the determination of structure.

1. Number of nucleic acid molecules per virus particle. From the molecular weight of tobacco mosaic virus (about 40×10^6) and an RNA content of about 5 percent (Knight and Woody 1958), it can be calculated that each TMV particle contains 2×10^6 daltons of RNA. Likewise, it can be calculated that a polynucleotide chain of about this molecular weight would just occupy the length of a 300-nm rod if it were located at the radius shown in Figure 18 and followed the helical pitch of the protein subunits,

as it appears to do (Franklin et al. 1959; Hart 1958; Schuster 1960a). The total length of such a fiber would be 3,300 nm.

The critical question, of course, is whether there is a single fiber of RNA or several molecules, perhaps subunits, regularly spaced along the length of the TMV particle. Studies on hot salt preparations of TMV-RNA, using light-scattering measurements, yielded a molecular weight of 1.7×10^6 daltons for the isolated nucleic acid (Hopkins and Sinsheimer 1955). Similarly, Boedtker (1959), starting from highly monodisperse preparations of TMV, obtained fairly homogeneous preparations of RNA by a modified hot salt method (dilute virus, 90° heating for 1–3 min), and the molecular weight reported for this RNA, as determined from both light-scattering and sedimentation-viscosity measurements, was $1.94 \pm 0.16 \times 10^6$. Other light-scattering investigations were made by Friesen and Sinsheimer (1959) on TMV-RNA prepared by either the detergent or phenol procedures. A weight average molecular weight of 2×10^6 was found for both types of preparation, and infectivity was associated with this material and not with the smaller components that appeared upon storage of the RNA.

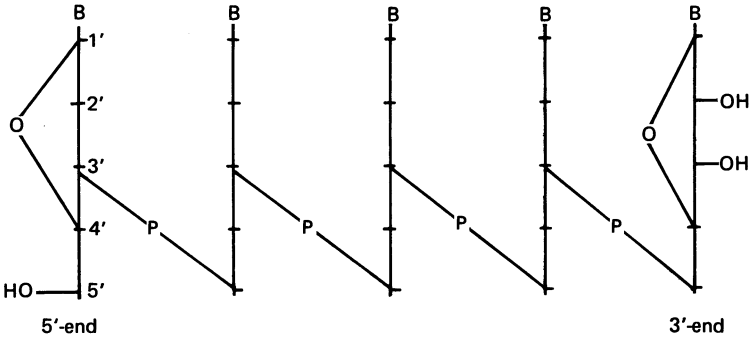
The physical properties of the RNA obtained by the phenol extraction procedure were also investigated extensively by Gierer (1957, 1958a, 1958b, 1958c). In sedimentation studies a well-defined, high molecular weight component was observed that accounted for the bulk of the RNA, the rest appearing on the sedimentation diagrams as smaller, polydisperse products. The observed sedimentation coefficient was about 31 Svedberg units and, after applying a viscosity correction, a molecular weight for the RNA of 2.1×10^6 was calculated. When such nucleic acid was treated with ribonuclease, the kinetics of degradation was found to be as expected for the random splitting of a single-stranded structure (Gierer 1957).

Thus, such data indicate that the RNA isolated from TMV has a molecular weight equivalent to the entire RNA content of a virus particle, and that it occurs in the form of a single strand. A question unanswered at this stage was: Is the RNA a uniform polynucleotide chain in which all the 6,400 nucleotides are linked by covalent bonds, or might there be polynucleotide subunits, joined perhaps by hydrogen bonds, to form a single strand? Gierer found (1959, 1960) that TMV-RNA could be heated at 70° for 10 min or at 40° in 36 percent urea for 30 min without the RNA strands breaking down. Since both of these treatments are known to be disruptive to hydrogen bonds, it may be concluded that these are not linking polynucleotide subunits together in TMV-RNA but TMV-RNA is rather a single, large, polynucleotide strand. More recently, studies of the electrophoretic migration of TMV-RNA in polyacrylamide gels yielded results consistent with a viral RNA strand of about 2×10^6 daltons (Bishop et al. 1967).

The RNAs of several phages as well as of animal viruses of the polio and mouse encephalitis types appear to occur in their respective virus particles in one piece, like TMV. The same holds true for DNA in a wide variety of viruses. However, there are some exceptions to the unitary genome struc-

ture. For example, the double-stranded RNA of reovirus occurs in 10 segments (Shatkin et al. 1968; Millward and Graham 1970). A similar situation is found with the double-stranded RNAs of cytoplasmic polyhedrosis and wound tumor viruses (Kalmakoff et al. 1969). In addition, the single-stranded RNA of influenza virus occurs in segments (Barry et al. 1970) and there may be other examples. Two consequences of a segmented genome are that it provides a basis for unusually high recombination in mixed infection (genotypic mixing) and it provides individual gene segments of nucleic acid that can probably be sequenced and the data subsequently related to specific gene products.

2. *End group determinations.* Names are given to the two ends of polynucleotide chains of nucleic acids just as they are for ends of the polypeptide chains of proteins. Thus, in formulas depicting nucleic acid structure, the left side of the linear array of nucleotides is customarily referred to as the 5′-end of the structure, while the right side is called the 3′-end. This nomenclature is based on the occurrence of a free hydroxyl on the 5′-carbon and 3′-carbon atoms, respectively, of the terminal nucleotides. This can be illustrated in the following abbreviated structure for RNA:



Inspection of this structure shows that the ribose residues (indicated by vertical lines topped with a purine or pyrimidine base, B) are joined by 3′-5′ linkage through phosphate (P). The 5′-OH is involved in this linkage in every case except for the terminal residue on the left. This terminal is therefore recognizable on the basis of its free 5′-OH as the 5′-end. Similarly, the 3′-OH is involved in the formation of phosphodiester linkages everywhere except in the ribose residue on the extreme right. The free 3′-OH there marks this residue as the 3′-end.

Two important features of the ends of nucleic acid chains are often investigated: the presence or absence of terminal phosphate groups and the nature of the purine or pyrimidine base on the terminal nucleotides.

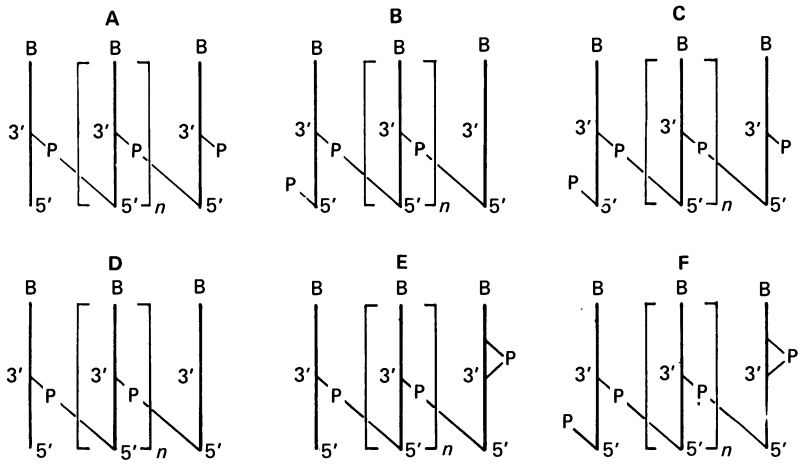


Fig. 19. Schematic representation of six chain end situations for RNA. Using the shorthand notation of Fig. 17*c* for the polynucleotide chain, B stands for purine or pyrimidine base, P for phosphate group, vertical lines for ribose residues, and *n* for number of nucleotides. (From Gordon et al. 1960.)

Some possibilities with respect to terminal phosphate groups, for example in TMV-RNA, are illustrated in Figure 19. It can be seen that treatment of RNA having a structure like that represented by A, B, or C, with an appropriate phosphatase enzyme (see Fraenkel-Conrat and Singer 1962), should release either one or two moles of inorganic phosphate per mole of RNA. By quantitative analysis of the released phosphate, which on a small scale is best done with ^{32}P-tagged RNA, the mean size of the RNA chains can be calculated. Subsequent mild alkaline hydrolysis, which breaks internucleotide links to the right of the phosphate groups as they are depicted in Figure 19, would yield one nucleoside per mole of RNA, and this can be separated from the nucleotides by paper electrophoresis and identified by comparison with standard compounds. Similarly, if the RNA should have one of the structures depicted by B and D, an estimate of the mean chain size and nature of the terminal residues can be obtained by determining the quantity and nature of nucleoside (and nucleoside diphosphate in the case of structure B) produced when the RNA is degraded with dilute alkali.

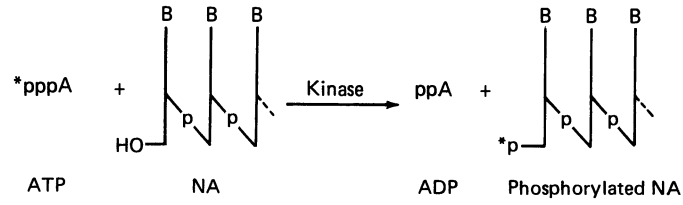
Other combinations of enzymatic and chemical treatments can provide information about the other end of the RNA chain, and such procedures can also be used to determine whether such structures as E and F are present. A structure similar to B except that it has a 5′-triphosphate group is common in certain phage RNAs. Alkaline hydrolysis of this structure would yield a nucleoside tetraphosphate that would have properties quite distinct from those of all the other digestion products and could be readily separated and identified.

The points just made regarding ends of RNA also apply in principle to DNA except that DNA is not subject to alkaline degradation and some of the enzymes active on DNA are different from those active on RNA. Also there is the problem of two strands in double-stranded DNA and these will need to be separated for some analyses. Finally, there is one structure not shown in Figure 19 that does occur with DNA, that is, a cyclic structure in which the two ends are joined. Essentially, in such a case, no split products are obtained when the nucleic acid is treated with phosphatases and exonucleases (exonucleases are phosphodiesterases that attack polynucleotide chains only or preferentially at ends and liberate mononucleotides therefrom. In contrast, endonuclease enzymes catalyze cleavage of mononucleotides from various locations in the middle of the chain). The cyclic single-stranded DNA of coliphage ØX174 constitutes such a structure (Fiers and Sinsheimer 1962a, 1962b) and cyclic double-stranded DNA is common in DNA-containing tumor viruses (Crawford 1968).

In the initial studies on TMV-RNA using ^{32}P-labeled RNA and treatment with prostatic phosphomonoesterase, Gordon et al. (1960) reported the release of one inorganic phosphate per 3,000–5,000 nucleotides. This was interpreted to mean that TMV-RNA had at the most one monoesterified end. Later results obtained with an *E. coli* phosphomonoesterase and specially purified RNA indicated considerably less than 1 mole of phosphorus per mole of RNA (Fraenkel-Conrat and Singer 1962; Gordon and Huff 1962). This combined with the evidence by Fraenkel-Conrat and Singer that snake venom diesterase (which catalyzes cleavage to the left of each internucleotide phosphate group in the formulations of Fig. 19) caused the release of considerably less than one mole of nucleotide diphosphate from TMV-RNA, leads to the conclusion that there is no monoesterified phosphate in TMV-RNA.

Assuming on the basis of the above that formula D of Fig. 19 represents the correct structure for TMV-RNA, it can be seen that mild alkaline hydrolysis should yield one nucleoside per mole of RNA from the 3′-end (right side of Fig. 19D), the rest of the RNA being converted to nucleoside 2′- or 3′-phosphates. Conversely, degradation of the RNA with venom diesterase should yield one nucleoside per mole of RNA from the 5′-end of the molecule, the rest of the RNA being converted to nucleoside 5′-phosphates.

However, the task of separating one nucleoside from approximately 6,400 nucleotides and identifying it is a formidable one. The key to the problem was the use of TMV-RNA highly labeled with ^{14}C (this is done by growing virus-infected plants in a chamber containing $^{14}CO_2$). When the degradative methods just outlined were applied to TMV-RNA, and the products were separated by paper electrophoresis and paper chromotography, it was found that in each case about 1 mole of adenosine was released per 2×10^6 daltons of RNA (Sugiyama and Fraenkel-Conrat 1961a,



1961b; Sugiyama 1962). Thus it appears that TMV-RNA has the structure represented by formula D of Figure 19 and that adenine is the base at both the 5′- and 3′-ends of the molecule.

However, it appears that the 5′-ends of TMV-RNA (and of brome mosaic virus RNA as well) may not be as uniform as the 3′-ends (Fraenkel-Conrat and Fowlks 1972). This was discovered by application of a neat labeling technique, that is, treatment of the nucleic acid with a polynucleotide phosphokinase (Richardson 1965), which transfers phosphorus from labeled adenosine triphosphate to the 5′-end of the nucleic acid:

Up to 1 mole of phosphate per mole of RNA was transferred in this manner to TMV-RNA, confirming the earlier conclusion that the 5′-end is unphosphorylated. The product was then degraded to nucleotides by alkali and the digest was analyzed for content of radioactive nucleoside 3′, 5′-diphosphates after separation of these by paper electrophoresis (or were analyzed for radioactive nucleoside 5′-phosphates separated from snake venom digests of the labeled RNA). Both methods indicated terminal heterogeneity with molar proportions of A:U:G:C = 54:17:18:11. As yet there is no evidence that this variability of the 5′-terminal of TMV-RNA has a serious effect on the biological activity, whereas oxidation of ribose in the 3′-terminal destroys most of the infectivity of the RNA (Steinschneider and Fraenkel-Conrat 1966).

The RNA of turnip yellow mosaic virus is another example of a viral nucleic acid with an unphosphorylated 5′-end (Suzuki and Haselkorn 1968). However, the 5′-ends of several phage RNAs have been found to terminate in triphosphate (Glitz 1968; de Wachter and Fiers 1969; Young and Fraenkel-Conrat 1970). Since nucleic acids appear to be synthesized from the 5′-end toward the 3′-end and nucleotide triphosphates are used in the syntheses, the presence of 5′-triphosphate ends as in the phage RNAs is expected. The absence of phosphate at the terminals of some of the plant virus nucleic acids may indicate that certain plant cells have more phosphatase activity than some other types of cells.

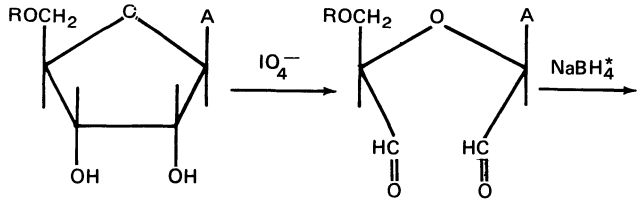
In any case, it appears that some viral nucleic acids as isolated from mature virus particles have phosphorylated ends and others do not. A special case of those that do not are the nucleic acids with cyclic structures.

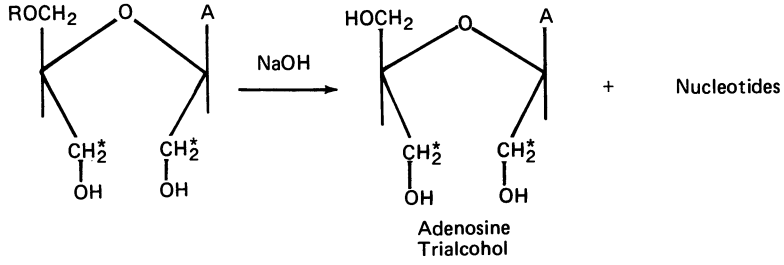
As just indicated, failure to detect release of significant amounts of phosphate when RNA is treated with *E. coli* phosphatase suggests that the terminals are not phosphorylated. This point can be checked with respect

to the 5′-terminal, and at the same time the nature of the terminal base can be established by phosphorylation of the 5′-OH of the terminal nucleoside using the Richardson kinase (Richardson 1965). Phosphorylation places a radioactive label on the terminal and subsequent alkaline hydrolysis yields one radioactive nucleoside diphosphate per RNA molecule and all the rest of the RNA as nucleoside monophosphates. The nucleoside diphosphate can be separated from the nucleotides and identified by electrophoresis (also called ionophoresis) in two dimensions (for details of methodology, see Dahlberg 1968; Brownlee 1972). Likewise, if the 5′-terminal is naturally phosphorylated, as those of several phage RNAs are, alkaline hydrolysis will yield a distinctive product that can be identified in the same manner. Uniformly ^{32}P-labeled RNA is highly desirable for this analysis because of the small amount of terminal compound compared with the bulk of the RNA. (Such compounds can be detected by ultraviolet spectrophotometric methods, but it should be noted that the sensitivity of the isotope technique employing ^{32}P is almost 1,000-fold greater than that with spectrophotometry.) Label can be introduced by growing virus in a medium containing ^{32}P-phosphate, or, if a specific replicase is available as it is for some phage RNAs, it is possible to synthesize a radioactive complementary copy by using unlabeled RNA as template in the presence of α-^{32}P-phosphate-labeled nucleotide triphosphates as substrates for the replicase (the three phosphate groups of nucleotide triphosphates are designated α, β, and γ, starting with α as the first one attached to the 5′-carbon).

Terminal 3′-groups can also be identified by special labeling procedures. An example of such a procedure for analyzing the 3′-end of a polynucleotide chain is the periodate oxidation-borohydride reduction method (Glitz et al. 1968; Leppla et al. 1968). This procedure requires an unphosphorylated terminal; if the end nucleoside is phosphorylated, the terminal phosphate is removed in a preliminary treatment with phosphatase. The adjacent 2′, 3′-hydroxyl groups on the ribose of the terminal nucleoside (these hydroxyls constitute the only such readily oxidized pair in the molecule) are oxidized to aldehyde groups by treatment with periodate. The aldehyde groups are next reduced and simultaneously tagged by treatment with tritiated borohydride. Finally, the terminal nucleoside derivative, conveniently referred to as a nucleoside trialcohol, is released by alkaline hydrolysis and separated from the nucleotides derived from the rest of the RNA molecule by paper or column chromatography procedures that permit its identification.

The main steps of the procedure can be illustrated as follows:





R stands for all of the RNA molecule except for the 3′-terminus, and A is adenine. The asterisk indicates location of the radioactive label (tritium). One atom of tritium is transferred from the borohydride to each alcohol group formed by reduction of the dialdehyde. A modification of this procedure that yields a somewhat less stable radioactive product is to substitute ^{14}C semicarbazide ($NH_2\text{-}NH\text{-}\overset{\overset{O}{\|}}{C}\text{-}NH_2$) for the tritiated borohydride (Steinschneider and Fraenkel-Conrat 1966), which then yields a radioactive semicarbazone.

Application of the method described above to TMV-RNA and to the RNAs of coliphages f2 and MS2 indicated that the 3′-terminal in all cases is adenosine (Glitz et al. 1968). The same result was obtained with the RNA of phage R17. But, as shown in Table 20, which illustrates the kind of data obtained, some distinctive situations were found with the double-stranded RNAs of three viruses with segmented genomes. Although analyses were made on individual segments of the genomes, the results were essentially the same within ± 2 percent; hence the table shows the results as though each virus contained only a single molecule of nucleic acid per particle as the R17-RNA does. A striking feature of the results shown in Table 20 is that the RNAs of cytoplasmic polyhedrosis and wound tumor viruses appear to have two different 3′-terminal nucleosides, uridine and cytidine. Since these appear in approximately equivalent amounts, it has been concluded that one strand of each RNA segment terminates in uridine while the complementary strand terminates in cytidine (Lewandowski and Leppla 1972).

Dahlberg (1968) has developed a procedure for analyzing the 3′-end of RNA that is uniformly labeled with ^{32}P. This procedure depends on the fact that in a complete T_1 ribonuclease (T_1 RNase) digest of RNA, the only product not susceptible to attack by alkaline phosphatase is the oligonucleotide derived from the 3′-terminal end of the RNA since this is unphosphorylated in most RNAs. (T_1 RNase catalyzes hydrolysis next to guanylic acid residues only. Oligonucleotide means a small polynucleotide segment.) Thus, a T_1 RNase digest of RNA is electrophoresed on DEAE paper providing a spread of oligonucleotide spots that are treated in situ with alkaline phosphatase. Charges will generally be different on the phos-

Table 20. The 3′-Terminals of some Viral RNAs as Determined from Incorporation of Label in Terminal Trialcohols Formed in the Periodate Oxidation-Borohydride Reduction Procedure.[a]

RNA from	Percent of Total Tritium Label in Nucleoside Trialcohols			
	U-triAlc[b]	G-triAlc	A-triAlc	C-triAlc
Reovirus	3		1	96
Cytoplasmic polyhedrosis virus	50		2	48
Wound tumor virus	52	1.5	6	40
R17 phage	3	1.5	94	1.5
Tobacco mosaic virus	1		99	

[a]Adapted from Lewandowski and Leppla 1972; Glitz et al. 1968.
[b]U-triAlc is uracil trialcohol, G-triAlc is guanine trialcohol, and so on.

phatase-treated oligonucleotides except for the terminal one. This will be the only one without any guanine, which migrates in the same way when subjected to electrophoresis at right angles to the first run but in the same solvent. The terminal oligonucleotide usually appears by itself on a diagonal drawn across the paper from the origin and can be eluted and digested separately with alkali, pancreatic RNase and venom nuclease. Electrophoresis in two dimensions of the digestion products provides data from which the nucleotide content and sequence can be deduced, including the 3′-terminal residue. Some data on terminal residues of a few viral RNAs are given in Table 21.

Table 21. Terminal Groups of Some Viral RNAs.

Virus	5′-End	3′-End	Reference[a]
f2, MS2, R17 coliphages	pppG. . .	. . .A_{OH}	1–3
Qβ coliphage	pppG. . .	. . .A_{OH}	1
Satellite necrosis	ppA. . .	. . .C_{OH}	4,5
Tobacco mosaic	A. . .	. . .A_{OH}	6,7
Turnip yellow mosaic	A. . .		8

[a](1) Dahlberg 1968; (2) Wachter and Fiers 1969; (3) Glitz 1968; (4) Wimmer et al. 1968; (5) Wimmer and Reichmann 1969; (6) Sugiyama and Fraenkel-Conrat 1961a; (7) Sugiyama and Fraenkel-Conrat 1962; (8) Suzuki and Haselkorn 1968.

h. Nucleotide Sequences

Viral nucleic acids, like viral proteins, are too large to analyze from end to end by stepwise degradation procedures. Methods of detecting struc-

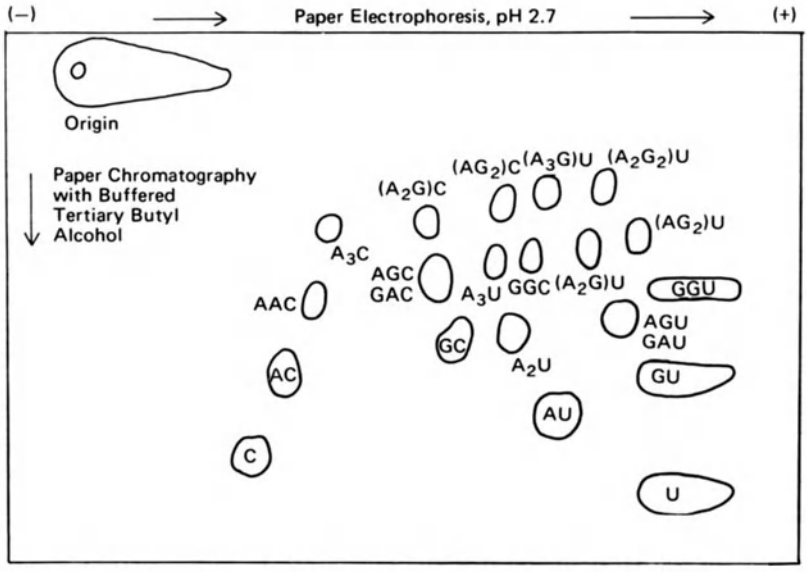
tural units (amino acids in the case of proteins and nucleotides in the case of nucleic acids) as they are split from the macromolecule are not sensitive enough to permit analysis of single molecules and hence thousands must be used. This requires substantial purity of the starting material and synchrony in the cleavage. Such synchrony can be achieved for a number of residues, but then begins to fail and is often accompanied by internal splits of the polynucleotide chain that yield spurious ends.

Consequently, the basic approach is to partially but specifically degrade the molecules into smaller fragments of various sizes. After purification, the sequences of these smaller fragments are established by further degradative procedures, often enzymatic, and identification of the products. By using enzymes of different specificities, and sometimes by partial rather than complete digestion, polynucleotide segments that overlap can be obtained. With these, progressively more of the oligonucleotides can be arranged in their correct order in larger segments of nucleic acid until the total sequence is deduced. Analyses of oligonucleotides for their nucleotide composition are made by alkaline and enzymatic digestion procedures.[2]

Some years ago, a two-dimensional procedure for separating small segments of RNA (one to ten nucleotides long) was developed (Rushizky and Knight 1960a, 1960b, 1960c; Rushizky et al. 1961; Rushizky 1967). The procedure was called two dimensional because it involves paper electrophoresis in one dimension on a large sheet of filter paper followed by chromatography in the second dimension on the same paper. The resulting reproducible spread of nucleotides and oligonucleotides on the paper was termed a "map" (some investigators subsequently called it a "fingerprint"). Such a map is shown in Figure 20. In the mapping procedure pancreatic ribonuclease (ribonuclease A) was employed at first to split the RNA into nucleotides and oligonucleotides. In subsequent experiments, a micrococcal nuclease (Reddi 1959; Rushizky et al. 1962a) and a fungal ribonuclease, ribonuclease T_1 (Miura and Egami 1960; Reddi 1960; Rushizky et al. 1962b), were used. An outline of the mapping procedure as it was applied to TMV-RNA is as follows.

To 1 ml of aqueous solution of TMV-RNA at about 8 mg/ml is added 0.3 ml of pancreatic ribonuclease (RNase) solution at 1 mg/ml and 0.02 ml of M sodium phosphate at pH 7.1. The mixture is allowed to stand at about 23° (room temperature) for 6–8 hr. To determine the precise amount of RNA being analyzed, two 25-μl aliquots are removed from the RNase digest, diluted with N KOH to 10 ml, and, after standing for 24 hr, read in the spectrophotometer at 260 nm. The initial concentration of RNA is calcu-

[2]Many procedures pertinent to analysis of oligonucleotides are described in detail in *Methods of Enzymology*, Vol. 12, Part A, Section II, L. Grossman and K. Moldave, editors, New York: Academic Press (1967).



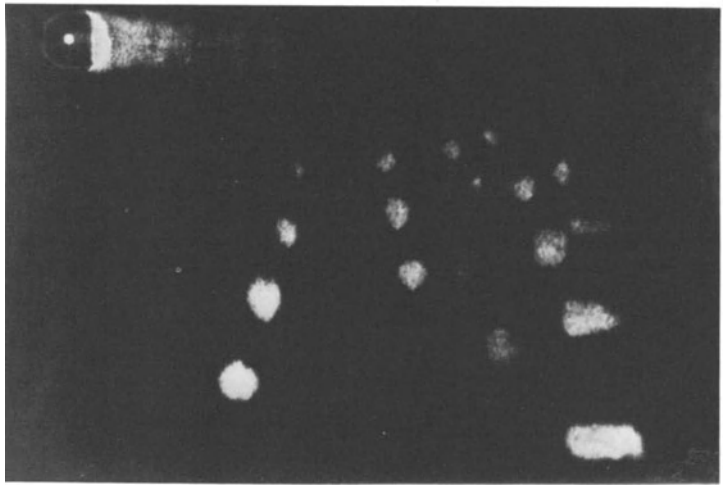


Fig. 20. Contact print map (*bottom*) and key to map (*top*) of pancreatic ribonuclease digestion products obtained from the RNA of the M strain of tobacco mosaic virus. (Maps for the RNAs of other strains and for many RNAs, including yeast RNA, show the same spots; however, they may differ in intensity, reflecting quantitative differences in runs of oligonucleotides). For convenience in labeling, the abbreviations used in the key omit the p normally used to designate phosphate groups. Thus Cp (cytidylic acid) becomes C, ApCp becomes AC, and so on. (From Rushizky and Knight 1960c.)

lated from the relationship that 1 mg of TMV-RNA/ml in N KOH has absorbance at 260 mn of 32.53 (Rushizky and Knight 1960c).

To fractionate the oligonucleotides in the RNase digest, 0.5-ml aliquots (equivalent to about 3 mg of RNA) are applied to buffer-moistened Whatman 3MM paper (46 × 57 cm), and a small application of picric acid (a visible electrophoresis marker that moves slightly faster than any component in the RNase digest) is also applied to the same edge but at the opposite corner of the paper. The buffer used is ammonium formate prepared by adding 7.0 ml of 98 percent formic acid to 2.5 liters of water and adjusting the pH to 2.7 with concentrated ammonium hydroxide. The paper electrophoresis is performed at 350 v (6 v/cm) for 17–20 hr with the point of application of sample near the cathode chamber. The run is finished when the picrate marker reaches the level of the buffer in the anode chamber. A Durrum-type electrophoresis apparatus was used by Rushizky and Knight (1960b). The paper is dried in a current of air at room temperature turned 90° from the direction used in electrophoresis, and serrated at the edge opposite the band of material in order to permit descending chromatography with runoff.

Chromatography is performed with a solvent consisting of equal parts of the electrophoresis buffer, adjusted to pH 3.8 with concentrated ammonium hydroxide, and tertiary butanol (the pH of the mixture at the glass electrode, without solvent correction, is about 4.8). For best results, chromatography should be carried out in a tank thoroughly saturated with solvent vapor for about 36 hr at approximately 25°.

After chromatography, the paper is dried in a current of air at room temperature, and the spots are located with an ultraviolet lamp. A record can also be secured by the contact printing method of Smith and Allen (1953), thus providing a map similar to that in Figure 20. Identification of the spots is by comparison with the positions of spots on a standard map on which the compounds had been identified by elution and analysis using enzymatic and chemical degradations (Rushizky and Knight 1960a).

For quantitative analysis, the spots, located under ultraviolet light, marked with a pencil, and cut out, are eluted in 5–10 ml of 0.01 N HCl at room temperature overnight. An appropriate paper blank is cut from each level of spots and treated in the same manner. The sample eluates are then read against an eluate of the proper paper blank in a spectrophotometer at 260 nm. The spectrophotometer readings are converted into quantities of compound by use of published extinction values for mononucleotides, or, in the case of the oligonucleotides, by use of extinction values calculated from composition using the extinctions of the component nucleotides. In such calculations, allowance is usually not made for the hypochromic state of the oligonucleotides since the error thus introduced is in most cases not great.

Some sequences found in TMV-RNA are summarized in Table 22. The

Table 22. Some Oligonucleotide Sequences Found in Tobacco Mosaic Virus Ribonucleic Acid.[a,b]

Dinucleotides	Trinucloetides	Tetranucleotides	Pentanucleotides
ApAp	ApApAp[c]	ApApApCp	(ApApApGp)Up
ApCp	ApApCp	ApApApGp	(ApApGpGp)Up
ApGp	ApApGp	ApApApUp	
ApUp	ApApUp	CpCpCpGp	
CpAp[c]	ApCpCp	UpUpUpGp	
CpCp	ApCpGp	(ApApCp)Gp	
CpGp	ApGpCp	(ApApGp)Cp	
CpUp	ApGpGp	(ApApGp)Up	
GpAp[c]	ApGpUp	(ApApUp)Gp	
GpCp	ApUpGp	(ApGpGp)Cp	
GpGp[c]	ApUpUp	(ApGpGp)Up	
GpUp	CpApGp	(ApCpCp)Gp	
UpAp	CpCpCp[c]	(ApCpUp)Gp	
UpCp	CpCpGp	(ApUpUp)Gp	
UpGp	CpUpGp	(CpCpUp)Gp	
UpUp	CpUpUp	(CpUpUp)Gp	
	GpApCp		
	GpApUp		
	GpGpCp		
	GpGpUp		
	GpUpUp		
	UpApGp		
	UpCpCp		
	UpCpGp		
	UpGpGp		
	UpUpGp		
	UpUpUp[c]		

[a]Data taken from Rushizky and Knight 1960c; Rushizky et al. 1961, 1962a, 1962b.

[b]The abbreviations are as indicated in the general section on nucleic acids. Where the composition is known but the sequence is not, parentheses are used.

[c]These sequences were deduced from higher oligonucleotides, whereas most of the compounds listed were actually isolated and identified as such after enzymatic digestion of TMV-RNA.

theoretical permutations (P) of the four common RNA nucleotides in which any nucleotide can occupy any position is given by $P = 4^n$ in which n is the number of nucleotide units in the oligonucleotide. Thus 4^2 dinucleotides are possible, 4^3 trinucleotides, and so on. As shown in Table 22, all 16 possible dinucleotide sequences have been found in TMV-RNA, as well as 27 of the possible 64 trinucleotide sequences. The compositions of relatively few tetranucleotide or higher fragments have been determined, although even now it is clear that a great variety occurs.

One of the findings arising from this early analysis of viral RNA sequences is that all RNAs yield the same pattern of oligonucleotides after complete digestion with pancreatic ribonuclease. However, definite quan-

titative differences are readily demonstrated, as will be described in the section on chemical differences between strains of a virus.

The determination of nucleotide sequences in a segment of nucleic acid larger than those just described for TMV can be illustrated by an example drawn from a study of the RNA of bacteriophage R17 (Jeppesen 1971). The nucleic acid of phage R17 was labeled by growing the bacteria infected with this virus in a radioactive medium, that is, a medium to which ^{32}P phosphate was supplied. The phage was isolated and purified by a combination of precipitation with ammonium sulfate and differential centrifugation. The RNA was isolated from the purified phage by extraction with sodium dodecyl sulfate and phenol, and analyzed by the following procedure.

About 20 μg of ^{32}P-labeled R17 RNA (approximately 20 μCi in radioactivity) is digested with 1 μg of ribonuclease T_1 for 30 min at 37° in 3 μl of 0.01M tris-HCl buffer, pH 7.4 containing 1 mM EDTA. The digestion mixture is then separated into oligonucleotides of various sizes and compositions by a two-dimensional procedure of electrophoresis and chromatography such as developed by Brownlee and Sanger and associates (see Brownlee 1972 for extensive details) and applied as follows.

The digest is applied to a 3 cm × 55 cm strip of cellulose acetate and subjected to electrophoresis in 7 M urea buffered with 5 percent (v/v) acetic acid and pyridine at pH 3.5 until the blue and pink marker dyes (xylene cyanol and acid fuchsin, respectively) separate by approximately 15 cm. The oligonucleotides, which can be detected by a portable Geiger counter, extend from the pink spot to about 3 cm behind the blue spot. The oligonucleotides are transferred from the cellulose acetate to a DEAE cellulose thin layer chromatography plate by placing the former on top of the latter and then a pad of Whatman 3 MM paper wet with water is placed on the cellulose acetate. The strips are pressed evenly together by placing a glass plate on top. Water from the paper pad passes through the cellulose acetate carrying the nucleotides with it into the DEAE cellulose where they are held by ion exchange.

Chromatography is performed on the thin layer plate by developing with a 3 percent mixture of partially hydrolyzed (10 min in 0.2 M NaOH at 37°) RNA dissolved in 7 M urea. (The use of carrier oligonucleotides in the chromatography of other oligonucleotides is called homochromatography.) The oligonucleotides from the partial digest of carrier RNA saturate the DEAE groups and displace the radioactive phage oligonucleotides. The latter then travel along in series of fronts with the nonradioactive oligonucleotides in accordance with size, which governs affinity for the DEAE groups. The smaller oligonucleotides are displaced by the larger ones and thus move more rapidly on the thin layer.

After the chromatography is completed, the thin layer is dried and the spots are located by autoradiography. The sort of separation achieved in

such a two-dimensional procedure is illustrated diagrammatically in Figure 21.

Oligonucleotides located by autoradiography can be individually removed from the thin layer and eluted from the DEAE cellulose with 30 percent (v/v) triethylamine carbonate at pH 10. The process of sequencing then involves treating aliquots of the isolated oligonucleotides with different enzymes, analyzing the resultant products, and deducing the sequence from the data obtained. The process of stepwise deduction of sequence can be illustrated for the oligonucleotide (a) (Figure 21) obtained from a ribonuclease T_1 digest of ^{32}P-labeled phage R17 RNA. Enzymes used to digest oligonucleotide (a) and the products found in each digest are summarized in Table 23.

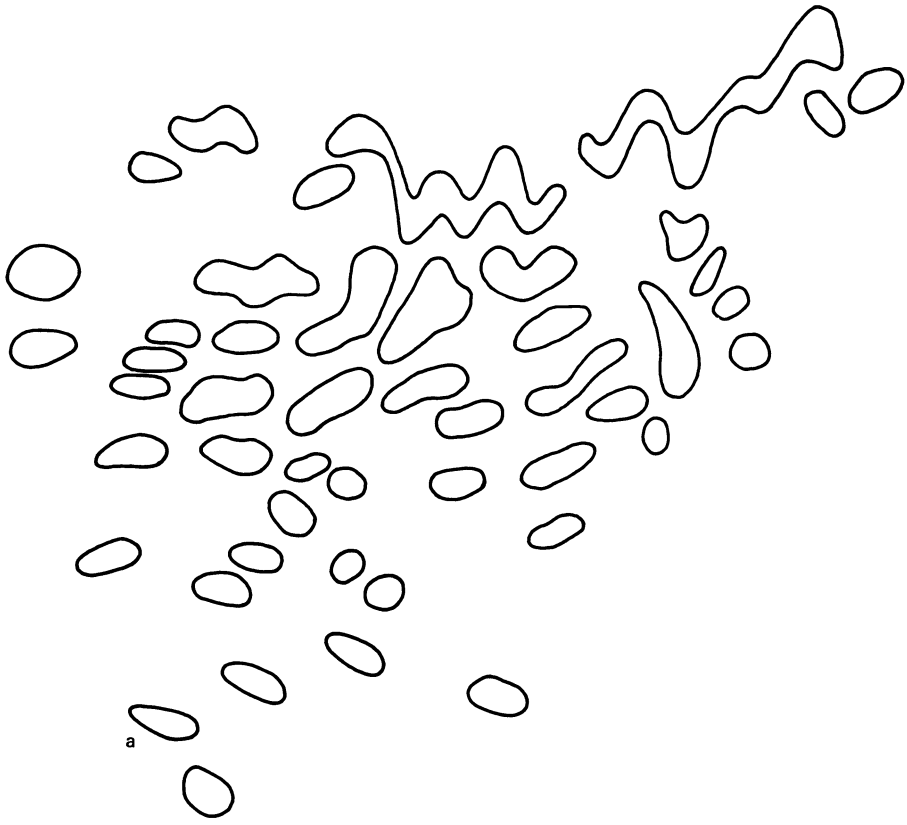


Fig. 21. Diagram of the spots revealed by autoradiography of a two-dimensional thin layer chromatogram. The material fractionated was a ribonuclease T_1 digest of ^{32}P-labeled phage R17 RNA. The oliognucleotide from the spot labeled (*a*) on the diagram is the one whose analysis is described in the text. (Adapted from Jeppesen 1971.)

Owing to the known specificity of ribonuclease T_1 (and confirmed by analysis) the 3′-terminal nucleotide of oligonucleotide (a) is Ġp, which is the only guanylic acid residue in the oligonucleotide. Since Ġp (see Table 23 for explanation of Ġp) was found in the ribonuclease A digest of the CD oligonucleotide in which only bonds next to cytidylic acid are split, the 3′-terminal sequence is -C-Ġp. A product of ribonuclease U_2 action is (U_4,C)Ġp, which enables extension of the deduced 3′-terminal sequence to Pu-U-U-U-U-C-Ġp, where Pu stands for purine. Since there is only one G in the oligonucleotide, all of the remaining purines must be A, and therefore the sequence is -A-U-U-U-U-C-Ġp.

Among the ribonuclease A products of the CD oligonucleotide (Table 23, column 2) there is $(A\text{-}A\text{-}U,U_3)$Cp, which, because it was derived by ribonuclease action limited to C residues, must be preceded by a C to give $(\text{-}C\text{-}A\text{-}A\text{-}U,U_3)$Cp. This sequence appears to overlap the previously de-

Table 23. Products Obtained by Enzymatic Digestion of Oligonucleotide (a) from Phage R17 RNA.[a]

Ribonuclease A Products[b] (Molar Proportions)	Ribonuclease A Products from CD Oligonucleotide (Molar Proportions)	Ribonuclease U_2 Products (Molar Proportions)
2 A-A-Up	1 (A-A-U̇,U̇)A-A-Cp	1(U_4,C)Gp
1 A-A-Cp	1(A-U̇,U̇$_2$)Cp	<1(U_2,C_2)A-Ap
1 A-Up	1 (A-A-U̇,U̇$_3$)Cp	<1(U_2,C_2)Ap
1 Gp	1 Ġp	<1 U-U-A-Ap
3 Cp	1 Cp	<1 U-U-Ap
6 Up		1(C,U)Ap
		1 A-Ap
		1 Ap

[a]Adapted from Jeppesen 1971.

[b]The respective enzymatic specificities yielding from oligonucleotide (a) the products listed in the three columns of the table may be summarized as follows. Ribonuclease A: The oligonucleotide is cleaved to the right of each *cytidylic acid* and *uridylic acid* residue. Ribonuclease A on CD oligonucleotide: A particular carbodiimide reacts with uridylic and guanylic acid residues to form carbodiimide derivatives (CD products) indicated by U̇ and Ġ in the Table. U̇ residues are resistant to digestion by ribonuclease A. Therefore, cleavages of CD oligonucleotide (a) occur only to the right of *cytidylic acid* residues. Ribonuclease U_2: This enzyme cleaves to the right of purine (adenylic and guanylic acid) residues, but purine-pyrimidine sequences are split more readily than purine-purine sequences. Partial splitting of purine-purine bonds accounts for the less than molar yields of four compounds in column 3 of the table. In the formulas of the table, the nucleotides are indicated by the first letter of their names and terminal phosphoric acid residues by p. The linkage of nucleotides by the conventional 3′-5′ phosphodiester bond is represented by a hyphen when the sequence is known and a comma when the sequence is unknown. Unknown sequences adjacent to known sequences are placed in parentheses. (These arrangements are in accordance with recommendations of the IUPAC-IUB Commission on Biochemical Nomenclature as summarized in *Journal of Molecular Biology*, **55**, 299–310, 1971.)

duced one and combining them yields -C-A-A-U-U-U-U-C-Gp. Looking for oligonucleotides that might overlap this sequence, it is observed that there are three ribonuclease U_2 products terminating in -A-Ap. Since none of the ribonuclease A products contain A-A-A, it appears that the A-Ap observed must come from the 5′-terminal of oligonucleotide (a). Of the remaining two ribonuclease U_2 products with terminal -A-Ap, only (U_2,C_2)A-Ap fits compositional requirements for overlapping the nine-nucleotide segment above.

The U_2C_2 can be arranged in six different ways (U-U-C-C, C-U-U-C, U-C-U-C, C-C-U-U, C-U-C-U, U-C-C-U) but only the three with C on the 3′-end would fit requirements for the overlap. Two of these possibilities are eliminated because they would have produced U-Cp or U-U-Cp upon degradation of CD oligonucleotide (a) by ribonuclease A and these compounds are not among the products. This leaves U-U-C-C as the proper sequence for U_2,C_2 and since the oligonucleotide containing this was obtained by action of ribonuclease U_2, it must be preceded by an A. This brings the known sequence at the 3′-end to -A-U-U-C-C-A-A-U-U-U-U-C-Gp.

The only remaining CD product which can overlap with this is (A-U,U_2)Cp whose sequence must therefore be U-A-U-U-Cp, which adds a U to the 3′-sequence just given. The CD oligonucleotide not yet appearing in the sequence is (A-A-U̇,U̇)A-A-Cp, which, in order to account for the three remaining ribonuclease U_2 oligonucleotides—A-Ap, U-U-A-Ap, and (C,U)Ap (the latter is involved in an overlap)—must have the sequence A-A-U-U-A-A-Cp. Therefore, the total sequence of the 21 nucleotides of oligonucleotide (a) from the above is A-A-U-U-A-A-C-U-A-U-U-C-C-A-A-U-U-U-U-C-Gp.

From this example it should be apparent that sequencing viral RNAs ranging from 3,200 to over 20,000 nucleotides is a formidable task and not likely to be undertaken even for the smallest viral RNAs unless the information to be gained is highly important. However, sequencing of small segments of RNA in order to elucidate initiation and termination signals for translation or to characterize enzyme attachment sites are projects that may warrant the effort required.

Analyses similar to those used for RNA have been employed to determine the sequence of the single-stranded ends (cohesive ends) of the mainly double-stranded DNA of lambdoid phages (phages similar to coliphage lambda) such as Ø80 (Bambara et al. 1973). It appears that the cohesive ends, comprising 12 nucleotides, of the DNAs of phages lambda and Ø80 are identical. This structural feature permits through base pairing the formation of interesting mixed dimers between the two phage nucleic acids. The sequences of the complementary strands are

(5′-3′): -A-G-G-T-C-G-C-C-G-C-C-C-OH
(3′-5′): HO-T-C-C-A-G-C-G-G-C-G-G-G-

A fragment of bacteriophage ØX174 DNA(a single-stranded DNA), 48 nucleotides long, has also been sequenced by using enzymatic and electrophoretic techniques similar to those used on RNA (Ziff et al. 1973). In this work good use was made of the T4-induced enzyme, endonuclease IV, which cleaves DNA to yield cytidine 5′ phosphate terminals.

i. Two Ways to Compare Nucleotide Sequences Without Sequencing: Nearest Neighbor Analysis and Hybridization

If there is an *in vitro* method available for the synthesis of nucleic acids from radioactive substrates (specifically, nucleoside triphosphates containing $\alpha^{32}P$), it is possible to compare nucleic acids in terms of the frequencies with which various nucleotide pairs (doublets) occur (since most nucleic acids contain four different nucleotides, the total number of different nucleotide pairs is $4^2 = 16$). This procedure, which has been termed "nearest neighbor analysis," was initially proposed by Josse et al. (1961).

The basic plan of the procedure is to use the nucleic acid whose analysis is desired as a template ("primer") for synthesis by a polymerase enzyme of complementary strands containing radioactive phosphorus at specific points in accordance with which radioactive triphosphate was used in the substrate mixture. Thus four successive syntheses are performed in which all four of the usual nucleotides are present, but in each case a different deoxyribonucleoside triphosphate contains the ^{32}P marker:

Reaction 1: ppp*A, pppG, pppC, pppT + template + polymerase
Reaction 2: pppA, ppp*G, pppC, pppT + template + polymerase
Reaction 3: pppA, pppG, ppp*C, pppT + template + polymerase
Reaction 4: pppA, pppG, pppC, ppp*T + template + polymerase

The mechanism of synthesis with DNA polymerase is that the substrates are 5′-deoxyribonucleoside triphosphates and these are linked into polynucleotide chains by esterification with the 3′-OH of adjoining nucleotides (see Figure 22). After synthesis is complete, the product is digested enzymatically to yield 3′-nucleoside monophosphates. In effect, the ^{32}P goes into the product in one nucleotide and comes out in digested product attached to the neighboring nucleotide (see Figure 22).

The nucleotides liberated by enzymatic digestion are separated by paper electrophoresis and the ^{32}P content of each is estimated in an appropriate counter. From the data the frequency with which any nucleotide occurs next to any other can be calculated. This then provides a measure of the frequency with which each of the 16 possible dinucleotides occurs in the nucleic acid in question.

This type of analysis appears to give patterns of dinucleotide frequency that are reproducible and characteristic for different nucleic acids and hence are useful in comparing nucleic acids. The examples given in Table 24 are drawn from the more extensive compilations of Subak-Sharpe et al.

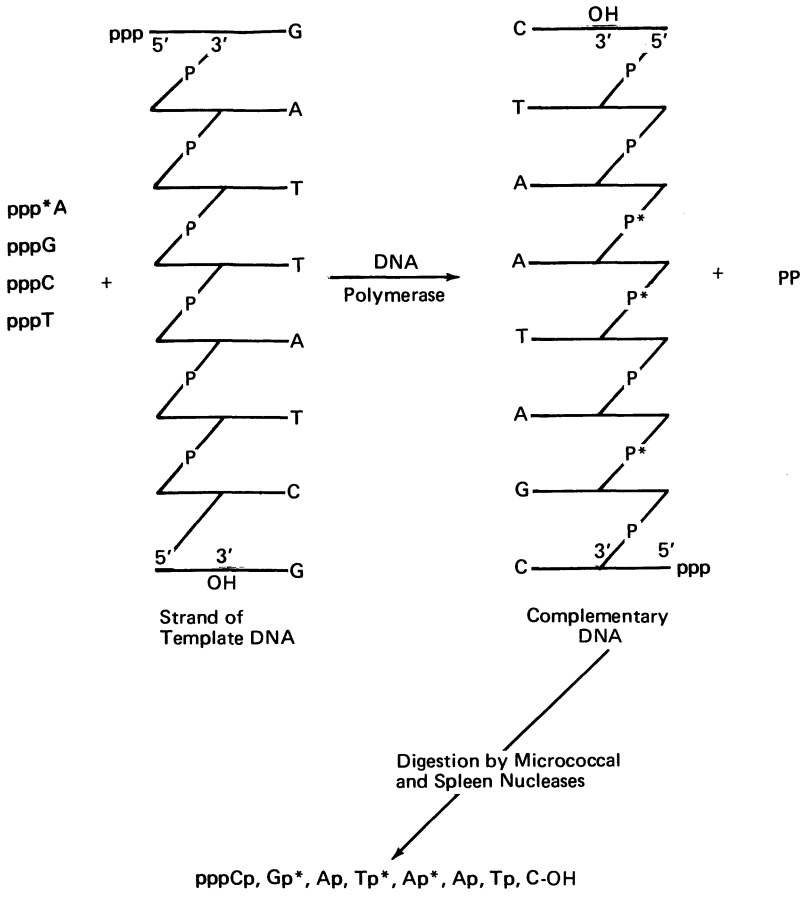


Fig. 22. Synthesis of new DNA from deoxyribonucleoside 5′ triphosphates. Radioactive phosphorus is indicated by p*. The new strand has a composition complementary to that of the template (when the template is double-stranded DNA, both strands serve as template for the polymerase). From the knowledge that each phosphate built into the new DNA is attached to the 5′ position of the entering nucleotide but leaves upon enzymatic digestion attached to the 3′ position of the neighboring nucleotide (the other partner of the phosphodiester linkage), the following dinucleotides can be deduced from the nature of the radioactive nucleotides in the enzymatic digest of the polymerase product shown above: GpA, TpA, and ApA.

Table 24. Some Doublet Frequencies[a] for DNA from Viruses and Other Sources.[b]

Doublet	Polyoma	Shope pap.	Vaccinia	Herpes	Phage λ	T1	T4	T5	ØX174	*E. coli* 2	Hamster BHK 21
ApT	56	57	69	68	70	64	64	65	59	72	53
TpA	50	45	58	68	47	48	55	62	46	47	50
GpC	64	70	54	65	72	76	70	78	80	72	75
CpG	22	30	72	65	64	70	53	58	59	64	13

[a]In parts per thousand normalized to correspond with DNA containing 50 percent (G + C).
[b]Adapted from Subak-Sharpe et al. 1966.

(1966). Even with only four of the 16 doublets represented in Table 23, it can be seen that there are substantial similarities between some of the T phages [Josse et al. (1961) found even closer correspondence, as would be expected on chemical, morphologic, and serologic grounds between the doublet frequencies for T2, T4, and T6], but also distinct differences among the several groups of viruses represented in the table. Comparison of the doublet frequencies of viral and host DNAs [see Table 24 and the more detailed results of Subak-Sharpe et al. (1966); and Bellett (1967)] leads to the conclusion that the smaller DNA-containing viruses exhibit doublet frequencies resembling those of mammalian animal cells (compare the values in Table 24 for the DNAs of polyoma and Shope papilloma viruses with those listed for hamster BHK 21 cells), whereas the DNAs of such large viruses as herpes and vaccinia, like those of phages, are more similar to bacterial DNA, such as that of *E. coli*. While the doublet frequencies do not provide adequate evidence to establish phylogenetic relationships, it has been suggested that the observed data are consistent with the idea that some bacterial and animal viruses evolved from their hosts, whereas others, such as herpes and vaccinia viruses may have had an external origin, perhaps from bacteria.

Limited nearest neighbor analyses have also been made on viral RNAs, including those of tobacco mosaic and turnip yellow mosaic viruses and of phage MS2 (Fox et al. 1964). Such analyses have also been especially useful in sequencing the RNA of some small phages (Bishop et al. 1968; Billeter et al. 1969).

In summary, comparisons of nucleotide doublets by the nearest neighbor technique can be very useful. However, such comparisons are in no way a substitute for the more arduous linear sequencing method outlined earlier. For example, as Josse et al. (1961) indicated, analytical errors as little as 1 percent, which are not unlikely, mean that even the moderate-size phage lambda DNA (molecular weight 20×10^6) could differ from a related DNA in 1,000 nucleotide sequences without any differences being detected by the nearest neighbor analyses.

The molecular hybridization technique is a method for obtaining infor-

mation about nucleic acid sequences that are very much longer than the doublets of the nearest neighbor method. The basis for this procedure is as follows.

Hydrogen bonds between complementary bases (A:T or U, and G:C) of the two strands of double-stranded nucleic acid (Figure 24) are broken by heating (and also by certain chemicals such as alkali or dimethyl sulfoxide), but the bonds are reformed by slow cooling ("annealing" or "renaturation") or removal of added chemical in the case of chemically induced destruction of hydrogen bonds. The separation of nucleic acid strands by heat is called molecular melting or denaturation, the latter being the more general term for strand separation by whatever cause. If strands of a second species of nucleic acid are added to those of the originally denatured one during the annealing process, strands of the second nucleic acid may compete with the original ones in the reforming of double stranded structures. This occurs only if substantial nucleotide sequences are the same or very similar in the two species of nucleic acid. If such homology exists, "hybrids" may be formed between complementary strands of DNA or between complementary strands of DNA and RNA; thus, the term hybridization is applied to the process.

Experimentally, either agar gels, or more commonly now, nitrocellulose filters, are used to immobilize denatured strands of nucleic acid. Hybrids can be formed if complementary structures are brought in contact with such fixed, denatured nucleic acid. (Appropriate nitrocellulose membranes bind denatured DNA, DNA-DNA, and DNA-RNA hybrids, but not free RNA or undenatured DNA.) In addition, there are procedures in which columns containing hydroxyapatite or other substances are employed. In almost all cases, quantitation is achieved by having one of the reacting species radioactively tagged. Details of various methods are given by Raskas and Green (1971) and Bøvre et al. (1971). An evaluation of the specificity of hybridization reactions is given by McCarthy and Church (1970).

Some examples of situations in which homologous sequences of nucleic acid or lack of them can be demonstrated by molecular hybridization include an evaluation of the degree of similarity of the nucleic acids of viruses that are thought to be similar, for example, comparison of the DNAs of the many types of adenovirus; investigation of the amount of replicating viral nucleic acid present at different times after infection; estimation of the number of viral genomes incorporated into host nucleic acid; determination of the presence or absence of virus-specific messenger RNA (mRNA); and discrimination between types of mRNA present at various times after infection.

An example involving simian virus 40 (SV40) can be cited here of the use of molecular hybridization to demonstrate the presence and number of copies of SV40 DNA integrated into the DNA of mouse cells (Westphal and Dulbecco 1968). Such integration of viral genome is thought to be as-

sociated with the transformation of normal cells to tumorous cells. In order to achieve greater sensitivity in the hybridization test, instead of seeking direct hybridization between viral DNA and mouse cell DNA, RNA complementary to SV40 DNA (cRNA) was employed. Two advantages of using cRNA rather than the direct approach with viral DNA are that higher specific radioactivities can be readily obtained in the cRNA and the use of cRNA eliminates the possibility of self-annealing of denatured viral DNA. Reconstruction experiments indicate that as few as three or four viral DNA molecules per cell can be detected by hybridization with cRNA (this is equivalent to less than two parts of viral DNA in a million parts of cellular DNA).

cRNA is synthesized *in vitro* from highly radioactive nucleoside triphosphates using SV40 DNA as primer and a DNA-dependent RNA polymerase. Hybridization is carried out essentially by the method of Gillespie and Spiegelman (1965). The circular, supercoiled SV40 DNA molecules are treated with deoxyribonuclease for 60 min at 30° to convert them into linear, circular strands with some breaks in one or another of the strands. This DNA is poured into 2 vol of boiling water and boiled for 15 min, thus effecting strand separation. The denatured DNA is chilled in ice and adjusted to contain 0.9 M NaCl–0.09 M Na citrate (called 6× SSC, 1 SSC being 0.15 M NaCl–0.015 M Na citrate). The DNA is then slowly passed through a Millipore membrane filter to which much of the denatured DNA attaches. In order to check the variability of results, replicate filters are employed with comparable aliquots of DNA.

The filters are next incubated at 66° for 22 hr in vials containing 1 ml 6X SSC, tritiated cRNA obtained as described above, 1 mg yeast RNA carrier, and sodium dodecyl sulfate at a concentration of 0.1 percent (the latter appears to reduce background counts by reducing nonspecific attachments of the radioactive cRNA). The filter-containing vials are gently shaken in a water bath during incubation. After incubation, during which hybridization is expected to occur when possible, the filters are removed from the vials and washed with 50 ml of 2X SSC using suction filtration. After this the filters are treated with 20 μg/ml of RNase A and 10 units/ml RNase T_1, for 60 min at 37°. Following this treatment, which is designed to remove RNA complexed to the DNA over short regions and hence of uncertain specificity, the filters are washed again, dried, and counted in a scintillation counter. The amount of DNA on each filter to which the counts need to be related is determined after counting by the colorimetric diphenylamine reaction.

In the investigation by Westphal and Dulbecco (1968) it was found that the DNAs of two different lines of mouse cells that had been transformed by SV40 (SV3T3-47 and SV3T3-56) fixed by hybridization different amounts of the SV40 specific cRNA. The counts per minute per 100 μg DNA above the backgrounds observed with the DNAs of untransformed

cells were, respectively, 800 and 264 (these are the means from counts of 10 to 12 filters each). From calibration tests in which known numbers of SV40 DNA molecules had been added to cell DNAs, it was determined that 40 cpm from attached cRNA was equivalent to one SV40 DNA; therefore, the counts per minute noted above represent about 20 and 7 SV40 DNA molecules, respectively, present in the DNAs of the two mouse cell lines that had been transformed by the virus. In these same experiments, it was demonstrated by hybridization that the SV40 DNA was in the nucleus rather than in the cytoplasm of transformed cells.

Another example may be cited, without giving the experimental details, to illustrate the many useful applications of molecular hybridization. Lacy and Green (1967) investigated the hybridization reactions between the DNAs of six members of the weakly oncogenic (oncogenic means tumor inducing) adenovirus group consisting of serological types 3, 7, 11, 14, 16, and 21. It was found that these viral DNAs are closely related, apparently sharing 70–100 percent of their nucleotide sequences. However, the DNAs of the weakly oncogenic adenoviruses apparently differ substantially from those of the strongly oncogenic types 12 and 18, for the results of the hybridization tests indicated that the two groups showed only 11–22 percent homology.

j. Secondary and Higher Structure of Nucleic Acids

The term "secondary structure" will be used here as it was in connection with proteins to mean geometric configuration, with special reference to the presence or absence of helical, hydrogen-bonded structures; similarly, folding of the polynucleotide chain can be considered "tertiary structure."

A secondary structure for DNA was proposed by Watson and Crick (1953a, 1953b) that seemed at once compatible with data on the composition of DNA, general chemical features of DNA, and x-ray diffraction data (Wilkins et al. 1953). The validity of this structure has been confirmed by many experiments over the succeeding years, and it is now widely accepted for DNA from many sources.

This DNA structure is briefly described as a dyad, or duplex, of right-handed helical chains each coiled around the same axis but with antiparallel nucleotide sequences (sequences running in opposite directions). Such an arrangement is shown diagrammatically in Figure 23. The two chains are held together by hydrogen bonding between complementary pairs of bases, one base of each pair being a purine and the other a pyrimidine. Thus, as shown in Figure 24, adenine pairs with thymine and guanine with cytosine. In terms of DNA composition, this should be reflected in A/T and G/C ratios of unity. It will be further noted (Figure 24) that two hydrogen bonds can readily form between adenine and thymine on adjacent strands, but three can form between guanine and cytosine.

One of the first examples of DNA to be shown to give an x-ray diagram consistent with the Watson-Crick structure was that of T2 coliphage (Wilkins et al. 1953). At first, the results of chemical analyses seemed not to support this conclusion. The double helix structure requires equimolar amounts of adenine and thymine and of guanine and cytosine for proper base pairing. Early analyses (Wyatt and Cohen 1952) showed T2 DNA to contain 33.2 moles adenine, 35.2 moles thymine, 17.9 moles guanine, and 13.6 moles 5-hydroxymethylcytosine per 100 moles of bases. The agreement between adenine and thymine was fair, but the guanine/5-HMC ratio was seriously off. With the development of the double helix theory, interest in analyses grew, which led to refinements in procedure, and in this case especially, a recognition of the lability of 5-HMC under common hydrolytic conditions. When these factors were adjusted for, both the A/T and G/5-HMC ratios were found to be close to unity (Wyatt and Cohen 1953). The molar equivalence of purines to pyrimidines in double-stranded viral DNAs is illustrated by many examples in Table 19.

The double helical secondary structure of DNA imparts noteworthy properties to the particle that distinguish it from a single-stranded structure. Primarily, the double-stranded structure has greater rigidity and order than the single-stranded one, and this is reflected in hydrodynamic behavior, optical properties, and chemical reactivity. Thus the transition from helical structure to the less ordered random coil structure assumed by separated strands (or vice versa, since the process is more or less reversible) can be followed by:

1. Sedimentation behavior (helical form sediments slower, that is, has lower sedimentation coefficient).
2. Viscosity (helical form is more viscous than random coil). The properties described in (1) and (2) apply to helical and denatured DNAs in dilute salt in the middle pH range; under these conditions, the separated strands of denatured DNA collapse to a globular form that sediments faster and has a lower viscosity than undenatured DNA. If conditions are employed to keep the separated strands of denatured DNA extended, they may sediment slower and have a higher viscosity than the helical structure (see Studier 1965).
3. Optical rotation (helical form has "handedness" so that it acquires optical activity above that inherent in its components, such as the sugar. Thus optical activity is proportional to helicity).
4. Ultraviolet absorption (helical form absorbs less than random coil at 260 nm because of stacking of bases. The state of reduced ultraviolet absorption characteristic of an hydrogen-bonded, ordered structure is called "hypochromicity." An increase in absorption is then called hyperchromy and a decrease, hypochromy).
5. X-ray diffraction (helical forms have distinctive x-ray diffraction characteristics, of which the absence of meridional reflections is outstanding).

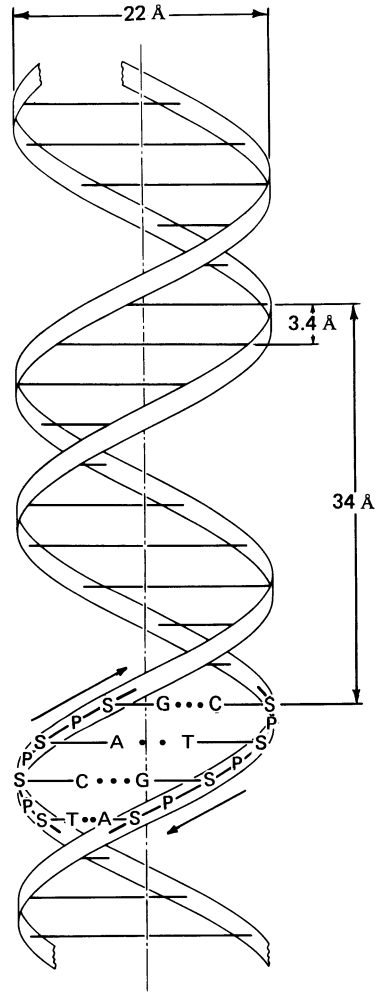


Fig. 23. Diagrammatic sketch of the structure of DNA (modified from Watson and Crick 1953b) by indication of the components in a segment: P, phosphate; S, sugar (2-deoxyribose); G, guanine; C, cytosine; A, adenine; T, thymine. The two ribbons represent the sugar-phosphate backbones of the two helical strands of DNA, which, as the arrows indicate, run in opposite directions, each strand making a complete turn every 34 Å. The horizontal rods symbolize the paired purine and pyrimidine bases. There are ten bases (and hence ten nucleotides) on each strand per turn of the helix. The nature of the base pairs and the number of hydrogen bonds between them are shown in the detailed central segment. The vertical line marks the fiber axis. (From Knight 1974.)

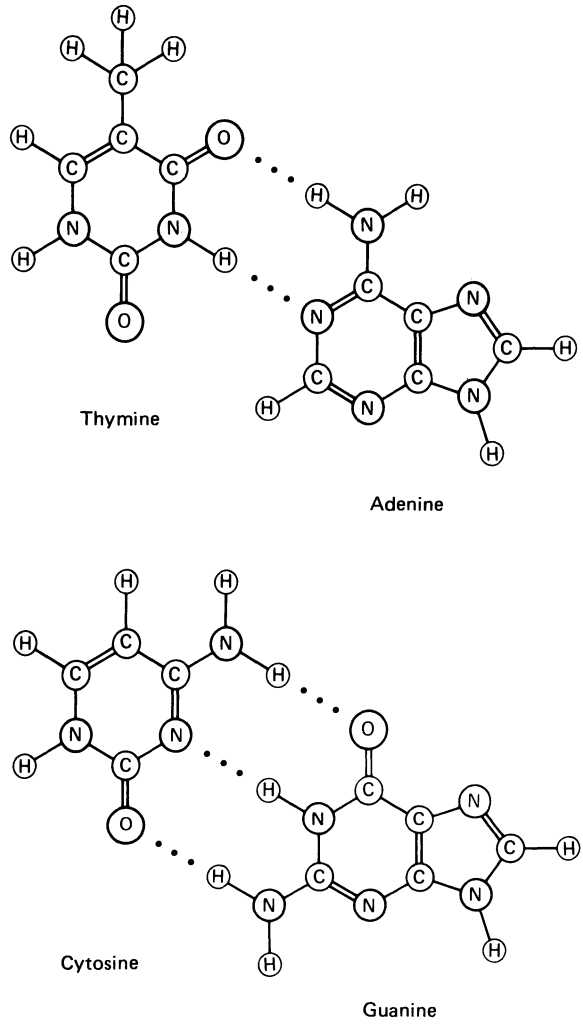


Fig. 24. Illustration of common hydrogen bonding that occurs between specific purine and pyrimidine bases in nucleic acids.

6. Chemical reactivity (keto and amino groups involved in hydrogen bonding do not titrate like free groups, and the reaction of amino groups with reagents like formaldehyde or nitrous acid is inhibited by hydrogen bonding).

7. Chromatography (double-stranded nucleic acid adheres to hydroxyapatite at salt concentrations and temperatures at which single-stranded molecules readily elute).

8. Electron microscopy (under appropriate conditions the thicker strands of double-stranded nucleic acid can be distinguished from those of single-stranded nucleic acid).

The nucleic acids of most viruses are linear structures but some, such as those of DNA tumor viruses and of some phages, are circular (see Table 16). The circular DNAs of animal tumor viruses tend to occur not only in circles but also in supercoiled forms or twisted circles. The significance of these unusual structures is not clear.

Turning now to RNA, it has been noted that the RNA of TMV is held in a helical configuration in the intact virus, and follows the pitch of the protein subunits at a radius of 40 Å from the long axis of the particle. In the TMV particle, the RNA therefore may be represented as a helix with a diameter of 80 Å and a pitch of 23 Å. Since this pitch is too large to permit hydrogen bonding between bases on successive turns of the helix, the helical structure must be stabilized simply by its position in the helical groove formed by the protein subunits (Klug and Caspar 1960). This situation is illustrated in Figure 18, a model drawing based on the results of the x-ray studies described earlier showing a segment of the TMV particle from which the protein subunits of the last two turns are removed. The nucleotides are indicated by the little discs in the RNA chain.

In contrast to the regular helical form assumed by TMV-RNA in the virus particle, the high molecular weight RNA isolated from the virus by the methods described earlier behaves in solution at low temperature and low salt concentration as though it were a flexible but tight random coil (Haschemeyer et al. 1959; Boedtker 1959; Gierer 1960) arising from a single polynucleotide strand. However, the strictly random coil concept is almost surely too simple to account for the observed physical properties of such isolated RNA in solution. The marked changes of properties of the RNA in different ionic media and at different temperatures strongly suggest the ready formation of secondary valence bonds under one set of conditions and the rupture of these bonds under another set (Boedtker 1959; Haschemeyer et al. 1959). Since there is no evidence for combination of separate RNA strands, it must be assumed that it is possible to form intramolecular bonds. To explore this possibility, Doty et al. (1959) applied to TMV-RNA some of the tests for helix-coil transition listed above.

In taking TMV-RNA in 0.1 M phosphate at pH 7 from 10° to 70°, Doty et al. (1959) found a 32 percent increase in absorption in the ultraviolet at 260 nm. This change was at least 95 percent reversible upon cooling. At room temperature, treatment of the RNA with 6 M urea caused half of the increase in absorbance observed in the thermal experiment.

In another test, the reaction of the RNA with formaldehyde at different temperatures was followed spectrophotometrically. This test was based on the observation by Fraenkel-Conrat (1954) that treatment of TMV-RNA with 1–2 percent formaldehyde at pH 6.8 caused a gradual increase in the ultraviolet absorbance at the maximum as well as a shift of 3–5 nm toward higher wavelengths. This effect seems to depend upon the presence of free amino groups in adenine, guanine, and cytosine, and is illustrated by the results shown in Table 25. In the experiments of Doty et al., an increase in reactivity of TMV-RNA with formaldehyde at 45° as compared with 25°

Table 25. Effect of Formaldehyde on the Ultraviolet Absorption of Viruses, Proteins, Nucleic Acids, and Some Nucleic Acid Constituents.[a]

Material	Approximate Increase of Maximum Absorption,[b] %	Approximate Shift in Wave Length of Maximum, nm
Nucleic acids and constituents:		
TMV-RNA (prepared by hot salt method)	+29	+3
TMV-RNA (prepared by detergent method)	+28	+3
Liver-RNA	+19	+4
Yeast-RNA (commercial)	+24	+3
Thymus-DNA	None	None
Adenine	+23	+5
Adenosine	+19	+5
Adenylic acid	+22	+5
Guanylic acid	+5	+5
Cytidylic acid	+16	+3
Thymine	None	+3
Uracil	None	+1
Uridine	None	None
Uridylic acid	None	None
RNA-Containing Viruses:		
TMV	+3	+3
TMV (after 24 hr in 1% sodium dodecyl sulfate)	+18	+3
Tomato bushy stunt virus	+15	+4
Turnip yellow mosaic virus	+15	+3
Tobacco ringspot virus	+23	+3
DNA-Containing Viruses and Proteins:		
Shope papilloma virus	None	None
T2-coliphage	−3	None
T2-coliphage (after 24 hr in sodium dodecyl sulfate)	None	None
TMV protein	None	None
Bovine serum albumin	None	None
Ovomucoid	None	None

[a]Adapted from Fraenkel-Conrat 1954.

[b]Solutions containing the equivalent of about 0.025–0.05 mg of nucleic acid per milliliter in 0.1 M phosphate buffer at pH 6–8 were treated with 1–2 percent formaldehyde for 12 hr at 40° or 48 hr at 23°. The same maximum was reached at the two time and temperature levels.

(after 50 min) was about 19-fold, whereas a control mixture of the appropriate nucleotides showed only a sixfold increase.

The results cited so far definitely support the concept that in dilute, neutral salt at low temperature, portions of the TMV-RNA chain are bound to other portions of the same molecule by hydrogen bonds, presumably of the base-pairing sort, but these results do not answer the question of whether the hydrogen bonding is random or occurs in definite regions in such a manner as to provide helical segments. Evidence for helicity was obtained by Doty et al. (1959) by studying the effect of temperature on the optical rotation of TMV-RNA. If the RNA were devoid of any regular secondary structure, that is, if it were a random coil, its only optical activity would be that of the ribose, which has asymmetric carbon atoms. However, if a significant portion of the RNA had helical structure, the contribution to optical rotation, as judged from known helical structures, might be substantial. Furthermore, such optical rotation should be largely abolished by treatments that convert helical structures to random coils. It was found that the specific rotation of TMV-RNA decreased about 160° in going from a temperature of 8° to about 75°. Most significantly, the optical rotation-temperature and optical density-temperature profiles can be shown to coincide by adjusting the ordinate scales as shown in Figure 25. Thus the decrease in optical rotation of TMV-RNA with rise in temperature is approximately congruent with the increase in optical density observed.

From these results, Doty et al. conclude that the hydrogen bonding in TMV-RNA occurs in definite areas and results in helical segments. Some support for such helical structure is provided by the x-ray pattern obtained by Rich and Watson (1954), which shows some of the characteristics of the patterns obtained with helical DNA. An estimate of the extent of these helical regions was made by Doty et al. by comparing the maximum variation with temperature of the specific rotation (or optical density) of TMV-RNA with the maximum variation observed with the completely helical model, polyadenylic acid-polyuridylic acid. The resulting conclusion is that about 50–60 percent of the nucleotides in TMV-RNA are involved in helical regions. It is further postulated, from experiments with polyribonucleotides and by analogy with DNA, that the predominant base-pairing is probably between adenine and uracil and between guanine and cytosine. Unmatched bases are also predicted and the resulting structure, illustrated in part in Figure 26, consists of a number of imperfect helical loops with randomly coiled regions interspersed. Such a structure would be compatible with the observed hydrodynamic properties of TMV-RNA and would especially account for variations in physical properties with changes in environment. A somewhat similar model has been deduced from the results of x-ray studies made on RNA from ascites tumor cells, *E. coli*, and yeast by Timasheff et al. (1961). They concluded that their RNA was represented by short, rigid, double helical rods about 50–150 Å long joined by small flexible single-stranded regions.

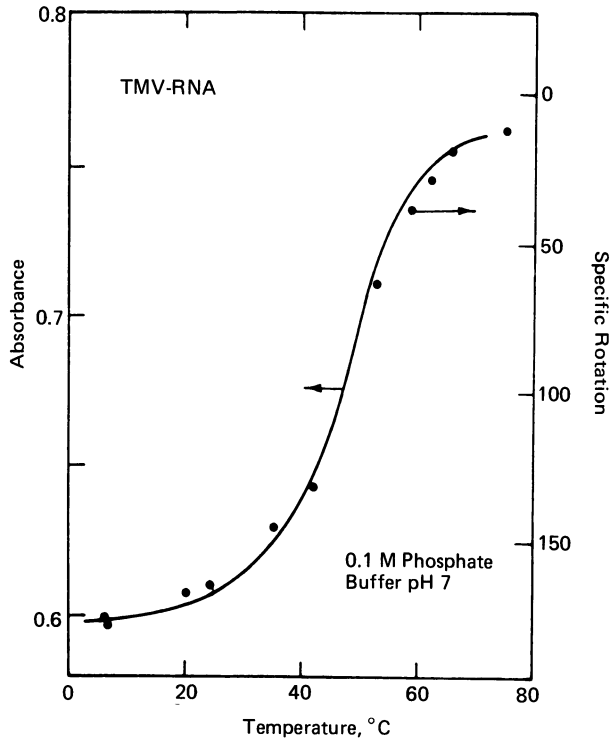


Fig. 25. The variation of specific rotation (*dots*) and adsorbance (*solid line*) of TMV-RNA with temperature. (From Doty et al. 1959.)

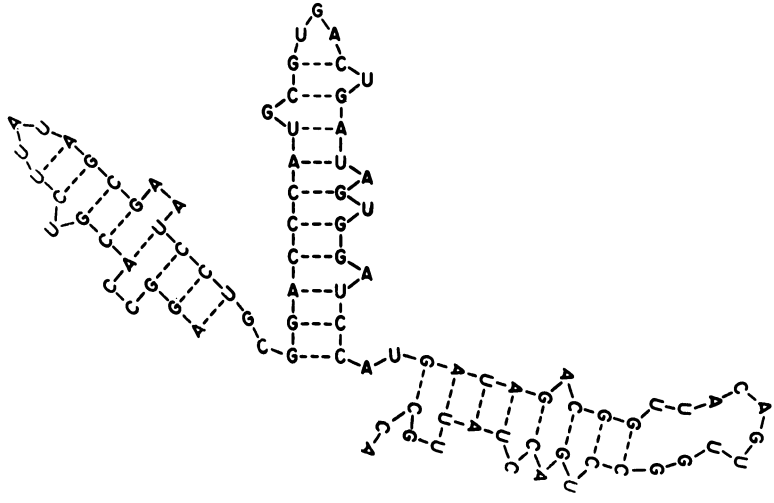


Fig. 26. A possible model for a segment of TMV-RNA in dilute neutral salt solution at low temperature. A, C, G, and U are abbreviations for the nucleotides: adenylic, cytidylic, guanylic, and uridylic acids, respectively. (From Doty 1961.)

Viral nucleic acids, whether single stranded, double stranded, or cyclic, generally appear to occur in unbroken strands. In contrast to this generality there are some RNA viruses whose nucleic acid appears to occur in segments. Thus the double-stranded RNA genomes of reovirus and of cytoplasmic polyhedrosis virus of the silkworm occur in ten segments (Shatkin et al. 1968; Millward and Graham 1970; Lewandowski and Millward 1971) and that of the wound tumor virus of clover in 12 segments (Kalmakoff et al. 1969; Reddy and Black 1973). (A viral genome may be defined as the total ensemble of genes associated with a virus.) Separation of the RNA segments of the cytoplasmic polyhedrosis virus by electrophoresis in acrylamide gel is illustrated in Figure 27. Similar segments but consisting of single-stranded RNA seem to characterize influenza viruses (Duesberg 1968; Pons and Hirst 1968), and possibly RNA tumor viruses (Vogt 1973).

It should be emphasized that, in general, the physical properties of nucleic acid within a virus particle may or may not be the same as those of the extracted nucleic acid treated as a hydrodynamic entity. This was demonstrated by Bonhoeffer and Schachman (1960) using four viruses, two of which contained DNA and two RNA. A comparison of the ultraviolet absorption spectra before and after degradation with sodium dodecyl sulfate with the spectra obtained by heating the degradation mixtures was used as a measure of the degree of hypochromicity (and hence of hydrogen-bonded structure) of the nucleic acid within the virus particles and upon release from them. In the case of the DNA viruses, Shope papilloma virus and T6 coliphage, no change in secondary structure upon release of the nucleic acid could be detected. Upon heating the degraded viruses, however, the ultraviolet absorptions increased 30–35 percent, from which it can be assumed that the DNA is present, in each of the cases, within the virus as well as upon release, in the form of the classical double helix.

On the other hand, a definite decrease in ultraviolet absorption occurred upon release of RNA from TMV, as shown in Figure 28. Heating the degradation mixture restored the absorption to the level of the undegraded virus. This result confirms the deduction made from the x-ray and other data (see above) that the spacing of RNA in the TMV particle precludes base-base interaction, which, however, occurs intramolecularly upon release of the nucleic acid. Thus the TMV-RNA appears to go from the protein-imposed helical configuration of the intact virus illustrated in Figure 18 to an intramolecularly, partially hydrogen-bonded structure such as shown in Figure 26.

Upon degradation of the bushy stunt virus, a slight decrease in absorbance was noted. Heating the degraded virus caused a 23 percent increase. From these facts it was concluded that the RNA within bushy stunt virus has some secondary structure and that more is acquired upon release of the RNA from the particle.

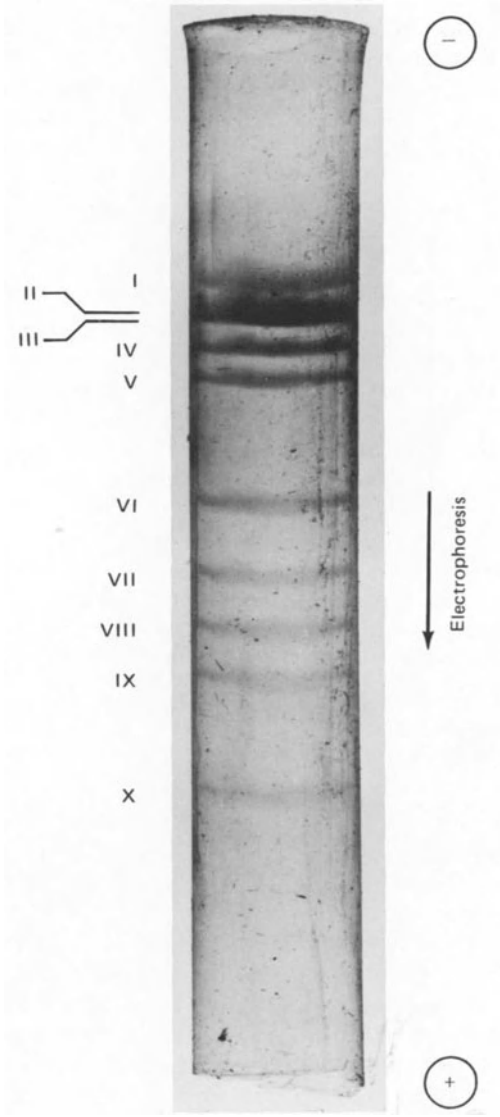


Fig. 27. Polyacrylamide gel electrophoresis of the double-stranded RNA genome of cytoplasmic polyhedrosis virus of the silkworm. There are ten segments of RNA, and all except segments II and III separated under the conditions of electrophoresis used for this experiment (3 percent polyacrylamide gel, pH 7.5, stained with methylene blue). (Courtesy B. L. Traynor.)

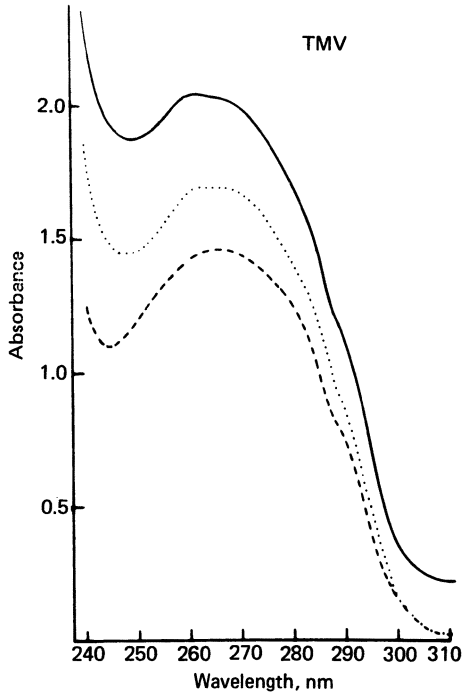


Fig. 28. Ultraviolet absorption spectra of TMV before and after degradation by sodium dodecyl sulfate into RNA and protein. Solid and dotted curves are for the intact nucleoprotein, the former giving the observed optical density values and the latter the values after application of light-scattering corrections. The dashed curve was obtained after degradation of the virus into RNA and protein. (From Bonhoeffer and Schachman 1960.)

Therefore, at present it appears that the nucleic acid within viruses can have (1) no independent secondary structure (TMV); (2) extensive secondary structure (Shope papilloma virus and T6); (3) partial secondary structure (bushy stunt virus).

Finally, it has been suggested by Fresco et al. (1960) that the unpaired bases in such semihelical RNA structures as illustrated in Figure 26 constitute a tertiary structure since they are held in loops or folds in fixed relation to the rest of the structure. It is further suggested that their conformation might provide new possibilities in coding and information transfer that are not inherent in a structureless single strand or in a perfect base-paired helix. These suggestions remain open for further investigation.

Investigations have also been made to determine whether the segmented state of certain RNA genomes mentioned above might be an artifact of preparation or represent the state of the nucleic acid in the viral particle as well. Application of the periodate oxidation-borohydride reduction pro-

cedure described in Sec. 2, g2 showed the same number of 3′ terminal groups for the RNA within the virus particles as in the isolated nucleic acid. Hence it appears that the nucleic acid in reovirus, cytoplasmic polyhedrosis virus, and influenza virus particles is segmented to the same extent as the nucleic acid isolated from such particles (Millward and Graham 1970; Lewandowski and Millward 1971; Lewandowski et al. 1971).

3. *Function of Viral Nucleic Acids*

That nucleic acids are the genetic material of viruses is now widely accepted. This view did not appear suddenly but evolved through a series of observations made over a period of years. The following are noteworthy examples.

a. A Suggestive Idea from Bacterial Transformation

In 1944 Avery et al. reported experiments in which it was shown that minute amounts of DNA extracted from Type III pneumococci were able, under appropriate cultural conditions, to induce the transformation of unencapsulated R variants (characterized by rough colonies) of pneumococcus Type II into fully encapsulated S cells (characterized by smooth colonies) of pneumococcus Type III. Evidence that the active transforming agent was actually DNA, unaided by protein, polysaccharide, or any other substance, mounted over the years, together with reports of transforming principles in other bacterial systems (see reviews by Zamenhof 1957; Hotchkiss 1957; Hotchkiss and Gabor 1970). Since the presence or absence of capsules was known to be genetially determined, the potential significance of DNA in bacterial genetics was made apparent by transformation phenomena. Moreover, other hereditary characteristics are now known to be transferred in this manner.

b. A Hint from the Chemical Analysis of Spontaneous Mutants of Tobacco Mosaic Virus

At a time when proteins were considered largely responsible for the biological properties of viruses, enzymes, and certain hormones, some mildly disturbing results were obtained upon analysis of the protein coats of spontaneous mutants of tobacco mosaic virus (Knight 1947a). At least one strain was found whose protein appeared to have the same composition as that of common TMV, although this strain caused markedly different symptoms in infected Turkish tobacco. Therefore, it was suggested that the primary change responsible for mutation might be in the nucleic acid of the virus. However, it was not certain that the amino acid analyses were accurate enough to have detected a small but significant difference between mutants (although this was later shown to be true), and there was the

possibility that the proteins of the two mutants were identical in composition but differed in sequence of amino acids. Consequently, these findings had less effect than might have been expected on the direction of thought about the role of viral nucleic acids.

c. RNA Shown Essential for Plant Virus Duplication

A few years after the work of Avery et al. (1944) on the pneumococcal transforming principle, Markham and Smith (1949) isolated and crystallized a new plant virus from turnip, which they called turnip yellow mosaic virus. This virus proved to be homogeneous in the Tiselius electrophoresis apparatus, but had two distinct components as judged from sedimentation studies. The major component, comprising 70–80 percent of the material by weight, and the minor component, comprising the balance of the material, had sedimentation coefficients of 106 S and 49 S, respectively. In other physicochemical properties, the two components were virtually identical save that the major component (called "bottom component" from sedimentation behavior) contained about 37 percent RNA, whereas the minor component ("top component") had essentially none. Significantly, the particles containing RNA were found to be highly infectious while those lacking RNA were noninfectious.

These findings could be interpreted to mean that only the combination of protein and nucleic acid is infectious, or that nucleic acid alone is essential for infectivity. Markham (1953) took the latter view in a paper presented at Oxford in April 1952, in which he said, "The role of the protein constituent of plant viruses is undoubtedly very important, but there is some evidence that the nucleic acid is in fact the substance directly controlling virus multiplication."

d. Role of DNA in Infection by T Phages

The idea of the hereditary primacy of viral nucleic acid received a great stimulus from Hershey and Chase's study (1952) of the process of infection by *E. coli* by coliphage T2. Using phage whose protein was labeled with ^{35}S and whose DNA contained ^{32}P, they showed that at least 80 percent of the phage sulfur (and hence most of the protein) remained on the outside of infected cells, whereas only 21–35 percent of the phosphorus (representing DNA) remained outside. The bulk of the protein was mechanically removed at this stage, and yet the cells went ahead and produced T2 phage. Furthermore, 30 percent or more of the parental phosphorus was found in the progeny phage in contrast to less than 1 percent of the sulfur. From these and other facts, it was proposed that the DNA probably exercises the genetic function of the phage, and the protein of a mature phage particle acts as a protective coat for the DNA and is responsible for the adsorption of the phage to the bacterium and the injection of the DNA into the cell.

e. Infectious Nucleic Acid from Tobacco Mosaic Virus

The crowning evidence that nucleic acid is the prime germinal substance of viruses was obtained when it was shown that TMV-RNA is infectious. This was demonstrated after the Hershey-Chase experiment, but a few years prior to the report that bacterial protoplasts (bacteria whose cell walls had been enzymically removed), in contrast to whole bacteria, could be directly infected with phage nucleic acid.

Fraenkel-Conrat (1956) reported that RNA preparations obtained from TMV by treatment of the virus with sodium dodecyl sulfate (see Detergent Procedure in section on Methods for Preparing Viral Nucleic Acids) was infectious though apparently devoid of characteristic virus particles, and that this infectivity was abolished by treatment of the preparation with ribonuclease. At about the same time, Gierer and Schramm (1956a, 1956b) described similarly infectious TMV-RNA preparations they had obtained by extracting the virus with phenol, according to the method of Schuster et al. (1956) (see The Phenol Method in the preparations section). The infectivity of the Gierer-Schramm preparations was also sensitive to ribonuclease; in addition it was shown to sediment much more slowly than virus, to be relatively unaffected by anti-TMV serum, and to be considerably more sensitive to elevated temperature than intact virus. Also, the level of protein in the infectious RNA preparations was found to be very low. These points were confirmed by Fraenkel-Conrat et al. (1957).

The indication from these pioneer experiments that viral nucleic acid is the genetic material of viruses has been repeatedly verified in many ways and is now taken for granted. Thus, in viruses, as well as in higher organisms such as protists, plants, and animals, a major function of nucleic acid is as a repository of genetic information. In addition, and in contrast with higher organisms, viral nucleic acid, when it is RNA, often acts as its own messenger RNA (mRNA). When viral nucleic acid is DNA, it cannot perform this function directly but serves as a template from which mRNA is transcribed. The nucleic acids found in mature virus particles, whether RNA or DNA, serve as templates for their own replication.

C. Lipids

Viruses containing lipid include representatives of all major types of viruses (see Table 26), although lipid components are much commoner among animal viruses than they are with bacterial or plant viruses.

Lipid-containing viruses share three common properties:

1. Particle morphology. The virus particles usually exhibit a nucleoprotein core surrounded by a membranous envelope composed of lipid, protein, and sometimes carbohydrate through which glycoprotein structures called spikes project (see Figure 31).

Table 26. Some Lipid-Containing Viruses.

Virus	Percent Lipid	Lipid Constituents	Reference[a]
Avian myeloblastosis	35	Partly phospholipid	1, 2
Equine encephalomyelitis	54	Phospholipids, cholesterol, triglycerides	3
Fowl plague	25	Phospholipid, cholesterol	4, 5
Fowlpox	27	Phospholipid, cholesterol, triglycerides, fatty acids	6
Herpes simplex	22	Phospholipid	7
Influenza	18	Phospholipids, tryglyceride, and cholesterol	8
Potato yellow dwarf	20	Phospholipids, sterol and possibly other lipids	9
Pseudomonas phage Ø6	25	Phospholipid	10
Pseudomonas phage PM2	10	Phospholipid	11
Rous sarcoma	35	Partly phospholipid	12
Simian virus 5 (SV5)	20	Phospholipids, cholesterol, triglyceride	13
Sindbis	29	Phospholipids, cholesterol	14
Tipula iridescent	5	Phospholipid	15
Tomato spotted wilt	19	Not yet known	16
Vaccinia	5	Phospholipid, cholesterol, triglycerides	17

[a](1)Bonar and Beard 1959; (2) Allison and Burke 1962; (3) Beard 1948; (4) Schäfer 1959; (5) Gierer 1957; (6) Randall et al. 1964; (7) Russell et al. 1963; (8) Frommhagen et al. 1959; (9) Ahmed et al. 1964; (10) Vidaver et al. 1973; (11) Espejo and Canelo 1968; (12) Vogt 1965; (13) Klenk and Choppin 1970; (14) Pfefferkorn and Hunter 1963; (15) Thomas 1961; (16) Best 1968; (17) Joklik 1966.

2. *Mechanism of maturation and release of virus particles.* Nascent virus particles mature at plasma, vesicular, or nuclear membranes through which they are then released by an extrusion or budding process.

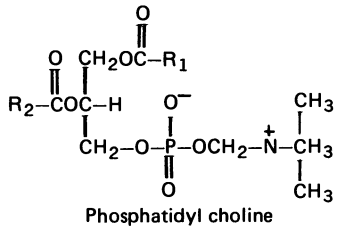
3. *Sensitivity to lipid-degrading agents.* Most lipid-containing viruses disintegrate and lose infectivity upon treatment with organic solvents (for example, ether or methanol-chloroform), certain detergents (for example, deoxycholate), or lipolytic enzymes (for example, phospholipase A). Vaccinia virus and certain iridescent insect viruses, which contain small amounts of lipid, are exceptions to this rule, but logical exceptions because their lipids serve little or no structural function, nor do they play a vital role in the infectious process.

Several different kinds of lipids have been identified among the fatty substances extracted from viruses and include cholesterol, triglycerides ("neutral fat"), and such phospholipids as sphingomyelin, phosphatidylcholine, phosphatidylserine, phosphatidylethanolamine, and phosphatidylinositol. The structures of some of these compounds are illustrated in Figure 29.

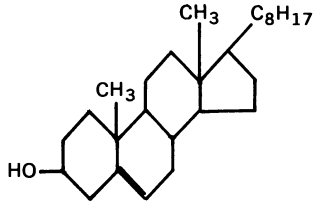
As noted above, most lipid-containing viruses have envelope structures that are acquired upon budding from a membrane. Abundant analytical evidence supports the assumption that much if not all of the viral lipid is obtained directly from the cell membrane in the budding process (Wecker 1957; Frommhagen et al. 1959; Kates et al. 1961; Franklin 1962; Klenk and Choppin 1969b, 1970). The chemical relationship between viral envelope lipids and cell membrane lipids is especially well illustrated by the studies of Klenk and Choppin (1969, 1970) with the paramyxovirus, simian virus 5 (SV5), and the membranes of different cells in which this virus was cultured. The data reproduced in Table 27 show how the quantities of different types of SV5 lipids parallel those of the membranes of two types of kidney cells in which the virus was grown.

Phospholipids predominate among the lipids found in cell membranes and this is reflected in the composition of viral lipids, including those of SV5 (Table 27). The comparison between cell membrane and viral lipids is sharpened by comparing the contents of individual phospholipids as is done for SV5 in Table 28. As shown in the table, there are marked differences in content of individual phospholipids between membranes from mouse kidney and hamster kidney cells. These differences are reflected in the compositions of SV5 lipids from virus grown in the two types of cells. Data of this sort mean that the same virus grown in different cell strains can have lipid of diverse compositions.

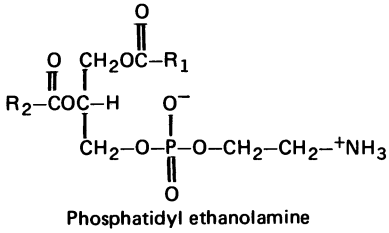
How are diverse lipids fabricated into viral envelopes? This is not yet clear. Cholesterol may be dissolved in the other fatty substances, but the various phospholipids appear to be coupled to protein and polysaccharide to form specific lipoprotein and glycolipid complexes. The precise nature of the linkages involved in these complexes and in their fabrication into an



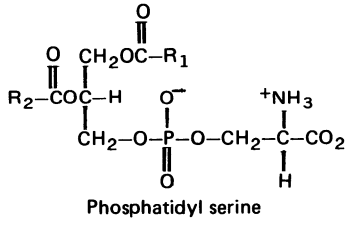
Phosphatidyl choline



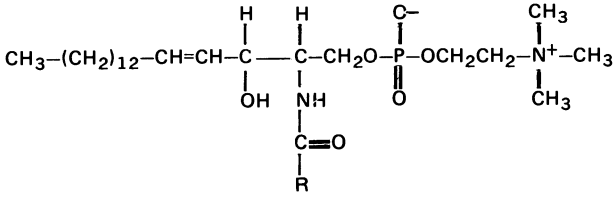
Cholesterol



Phosphatidyl ethanolamine



Phosphatidyl serine



Sphingomyelin

Fig. 29. Structural formulas for some phospholipids (phosphatides) and cholesterol. In these formulas R_1 is typically the hydrocarbon chain of a saturated fatty acid, while R_2 is a similar chain for an unsaturated fatty acid. Usually these fatty acids contain 16 or 18 carbon atoms.

Table 27. Lipid Content of Simian Virus 5 (SV5) and of Plasma Membranes from Monkey Kidney (MK) and Baby Hamster Kidney (BHK21-F) Cells in Which the Virus Was Grown [a]

Source	Total Lipid Percent of Dry Weight	Percent of Total Lipid			
		Phospholipid	Triglycerides	Cholesterol	Cholesterol Esters
MK membranes	28.5	55.0	5.1	23.0	0.3
SV5 from MK cells	20.0	50.9	5.0	29.0	0.9
BHK21-F membranes	30.7	60.0	2.8	20.5	1.3
SV5 from BHK21-F cells	21.0	57.0	3.0	18.6	2.7

[a]From Klenk and Choppin 1969b.

Table 28. A Comparison of the Phospholipid Contents of Simian Virus 5 (SV5) with Those of Plasma Membranes of Monkey Kidney (MK) and Baby Hamster Kidney (BHK21-F) Cells.[a]

Source	Percent of Total Phospholipid				
	Sphingomyelin	Phosphatidyl Choline	Phosphatidyl Inositol	Phosphatidyl Serine	Phosphatidyl Ethanolamine
MK membranes	11.8	32.1	—[b]	17.2	38.8
SV5 from MK cells	12.2	25.2	2.9	17.9	40.3
BHK21-F membranes	24.2	49.5	10.0	5.1	11.2
SV5 from BHK21-F cells	30.0	38.5	10.5	5.2	15.6

[a]From Klenk and Chopin 1969b.
[b]None detected.

envelope remains to be elucidated, but probably involves some of the same sorts of interactions that characterize enzyme-substrate complexes. In short, there is little evidence for primary covalent linkages between protein and lipid moieties in lipoproteins and the combination seems to depend on steric fit and upon interactions between nonpolar hydrophobic residues and between polar or charged groups. In addition there is probably significant hydrogen bonding in which water molecules have a bridging function.

1. *Preparation of Viral Lipids*

Lipids tend to be less soluble in aqueous media than the other constituents of viruses and more soluble in organic solvents. Generally, they are also more susceptible to air oxidation and to temperature effects. Consequently, lipids are extracted from frozen-dried (lyophilized) virus samples with organic solvents at moderate temperatures and often in an atmosphere of nitrogen. It should be noted that some lipolytic enzymes are solvent activated, an effect that increases with temperature. This suggests that extraction at room temperature is generally desirable.

Since in many cases lipids appear to occur in lipoprotein complexes and water plays some part in this union, it appears that dehydrating organic solvent should help to rupture the lipid-protein linkage. Hence such polar solvents as methanol and ethanol are usually included in the initial solvent of a several step procedure. However, since many lipids are not very soluble in such solvents, a more nonpolar solvent such as chloroform or diethyl ether is often included. A commonly employed solvent system of this sort is chloroform-methanol, approximately 2:1 (v/v). An example using such a mixture in the extraction of lipid from SV5 is as follows (Klenk and Choppin 1969a).

In order to extract total lipid, lyophilized virus is extracted with chloroform-methanol-water (65:25:5) (10 ml/50 mg dry weight) twice for 20 min at room temperature and once for 20 min under nitrogen using boiling solvent. To the combined extracts is added 1/6 vol of water, and the mixture is separated into aqueous and organic phases. If gangliosides (complex lipids composed of sphingosine, fatty acid, one or more sugars, and neuraminic acid) are present, they go into the aqueous phase while all other lipids remain in the organic phase. The latter includes virtually all of the SV5 lipid and the solvent in this fraction is removed under nitrogen in a rotary evaporator to give total lipids.

2. *Analysis of Viral Lipids*

The analysis of lipids obtained in the manner described in the previous section is fairly complex. The techniques employed include column chro-

matography, thin layer chromatography, gas-liquid chromatography, phosphorus analysis, and sometimes infrared spectroscopy. A detailed description of such methodology is given by Kritchevsky and Shapiro (1967); the details of the work on SV5 are described by Klenk and Choppin (1969a). A summary sketch of the SV5 analysis follows.

A sample of the total lipid fraction, extracted from SV5 as described in the previous section, was applied to a silicic acid column. Neutral lipids (a term applied to cholesterol and its esters, free fatty acids, and triglycerides) were separated from phospholipids by elution first with chloroform, which yielded neutral lipids, and then with methanol, which eluted phospholipids. The chloroform eluate was evaporated and the residue dissolved in hexane, which then was applied to a Florisil column (Florisil is a synthetic magnesium silicate). Chromatography on the Florisil column separated components of the neutral lipid, mainly cholesterol and its esters and triglycerides. The triglycerides were identified and quantitated as hydroxamic acids while cholesterol and its esters were also determined colorimetrically by another procedure. The methanol eluate from the silicic acid column was dried and dissolved in 2:1 chloroform-methanol. Aliquots of this solution were used for phosphorus determinations, and the different phospholipids were identified and quantitated by a combination of gas chromatography and quantitative two-dimensional thin layer chromatography. The results of such analyses are summarized in Tables 27 and 28.

3. *Function of Viral Lipids*

The lipids occurring in viral envelopes have been termed "peripheral structural lipids" (Franklin 1962). Extraction of these lipids with organic solvents or detergents or digestion of them with lipases results in considerable degradation of the viral particles. Clearly, such lipid components are essential for maintaining the structure of virus envelopes. The reason for loss of infectivity when viral lipids are removed is doubtless associated with the inability of disrupted virus to attach and penetrate because these steps in infection depend largely on viral surface structures. Specific attachment is especially important in infections by animal and bacterial viruses. How removal of lipid alters the infectivity of plant viruses is not yet apparent.

D. Carbohydrates

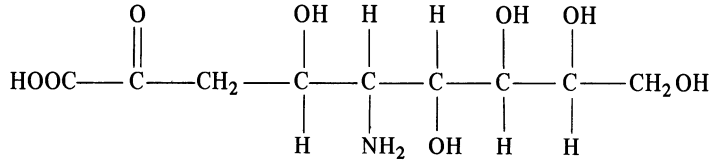
Carbohydrates are found in all viruses since all viruses have a nucleic acid component. Nucleic acids, as indicated in a previous section, contain one of two carbohydrates, ribose or deoxyribose. However, some viruses also contain nonnucleic acid carbohydrate. This has been observed in two

general situations: (1) glucose residues attached to pyrimidine in the DNA of certain bacterial viruses, and (2) polysaccharide coupled with protein (that is, glycoprotein) and lipid (glycolipid) in the envelope structures possessed by some animal and plant viruses. Quite a few enveloped viruses exhibit surface projections called spikes, and these are often glycoprotein in composition, especially in the case of viruses showing the capacity to agglutinate red cells.

Carbohydrate appears in the T-even (T2, T4, T6) bacterial viruses in the form of glucose or gentiobiose in O-glycosidic linkage with the 5-hydroxymethylcytosine (5-HMC) of the viral DNA (Sinsheimer 1960; Lehman and Pratt 1960; Kuno and Lehman 1962). The T-even phages contain essentially the same quantity of 5-HMC in their DNA components; yet the amount of glucoside is distinct for each. Glucose occurs in the proportions of about 0.8, 1.0, and 1.6 moles per mole of HMC for T2, T4 and T6, respectively (Jesaitis 1956; Lichtenstein and Cohen 1960). The glucose is uniformly distributed in the case of T4 where each HMC is glucosylated, but in T2 there is some unsubstituted HMC, some monoglucosylated, and a small amount of diglycosylated-HMC, the latter represented by the glucose disaccharide gentiobiose. In T6, an unsymmetrical distribution of glycosyl units is also found, but here about two-thirds of the glucose is present as gentiobiose.

There is evidence that the degree of glucosylation of the T phage DNA is an inherited trait, although in crosses the trait "glucose content" does not segregate symmetrically as a simple Mendelian character (Sinsheimer 1960). For example, the progeny of a T2 and T4 cross were all found to have the T4 glucose content, whereas the recombinants of a T2 × T6 cross were found to have the glucose content of either T2 or T6, although many more were found to have the glucose content of T2 and the host range of T6 than vice versa.

As indicated above, it has been found that viruses with an envelope structure often contain some of their proteins in the form of glycoproteins. Also, some of the lipid of viral envelopes may be present as glycolipid. The widespread occurrence of such nonnucleic acid carbohydrate constituents among viruses is illustrated by examples given in Table 29. In the table specific viruses are listed but in each case the group of viruses to which the example belongs is also indicated because the characteristics of the example are likely to apply throughout the group, and some of the groups are very large. The carbohydrate components of viral glycoproteins and glycolipids are complex polysaccharides usually fabricated from fucose, galactose, glucosamine, and mannose. Some viral glycoproteins, such as those from Sindbis and vesicular stomatitis viruses, contain sialic acid (Burge and Huang 1970). Sialic acid is the group name for a series of acylated derivatives of neuraminic acid:



Neuraminic Acid

(5-amino-3,5-dideoxy-D-glycero-D-galactononulesonic acid)

The simplest sialic acid is *N*-acetylneuraminic acid.

Although detailed structural analyses are yet to be made of viral glycoproteins, it seems likely, by analogy with other better studied glycoproteins, that the mode of linkage between protein and carbohydrate is by glycosidic bonds between carbohydrate chains and asparagine, serine, and threonine residues of the protein (Neuberger et al. 1972).

1. Preparation of Viral Carbohydrates

General methods have not been developed for the isolation of viral carbohydrates as they have for viral nucleic acids and proteins. Instead, it

Table 29. Some Viruses Containing Nonnucleic Acid Carbohydrate.

Virus	Carbohydrate-containing Constituent		Reference[a]
	Glycolipid	Glycoprotein	
Herpes simplex virus (a herpesvirus)	?	+	1
Influenza virus (an orthomyxovirus)	+	+	2
Murine leukemia virus (an oncornavirus)	?	+	3
OC 43 (a human coronavirus)	?	+	4
Potato yellow dwarf virus (a plant rhabdovirus)	?	+	5
Simian virus 5 (SV5) (a paramyxovirus)	+	+	2
Sindbis virus (a togavirus)	−	+	6
Vesicular stomatitis virus (an animal rhabdovirus)	+	+	7

[a](1) Roizman and Spear 1971; (2) Compans and Choppin 1971; (3) Nowinski et al. 1972; (4) Hierholzer et al. 1972; (5) Knudson and MacLeod 1972; (6) Schlesinger and Schlesinger 1972; (7) Knudson 1973.

has usually seemed sufficient to obtain qualitative and quantitative values for these constituents, and even such analyses have often been neglected. However, a growing realization of the importance of glycoproteins in many animal viruses may lead before long to the development of general procedures for isolating viral carbohydrates in undegraded forms.

The investigation of avian tumor virus glycopeptides represents a step in this direction. For example, Lai and Duesberg (1972) used the following technique to isolate avian tumor virus glycopeptides which were estimated to contain less than 10 percent protein: Purified tumor virus was disrupted by treating it at 37° for 30 min with 1 percent sodium dodecyl sulfate in the presence of 0.05 M mercaptoethanol. A precipitate of proteins and glycoproteins from the disaggregated virus was obtained by addition of 5 vol of ethanol; this precipitate was subsequently dissolved in a solution of 0.1 percent SDS–0.1 M tris buffer at pH 8. Alternatively, the viral proteins and glycoproteins were extracted from the virus by treatment with water-saturated phenol; they were recovered from the phenol phase by precipitation with 5 vol of ethanol in the presence of 2 M ammonium acetate. After washing twice with 75 percent ethanol, the precipitate was dissolved in the tris buffer–0.1 percent SDS noted above. By digesting the proteins in this mixture with pronase (a 48-hr treatment with pronase at 1 mg/ml followed by a second 48-hr treatment with pronase at 0.5 mg/ml) the proteins, including those of the glycoproteins, were largely degraded. The glycopeptides could be separated from this mixture by gel filtration chromatography on Sephadex G-50. When the viral protein had been labeled with ^{3}H amino acids before applying the procedures outlined above, it was found that over 90 percent of the label eluted from the Sephadex as amino acids or small peptides rather than in the glycopeptide fraction. This suggests that the procedure just described could be employed to prepare viral polysaccharides containing only a small amount of protein still attached.

2. *Analysis of Viral Carbohydrates*

The results of chemical and spectrophotometric analyses made by Taylor (1944) on influenza viruses provided the first indication that any highly purified virus contained nonnucleic acid carbohydrate. These early assays also indicated that the viral carbohydrate might contain galactose, mannose, and glucose. Later, more extensive studies based on colorimetric, chromatographic, and spectrophotometric analyses indicated that galactose, mannose, glucosamine, and fucose are constituents of the influenzal carbohydrates (Knight 1947b; Ada and Gottschalk 1956; Frommhagen et al. 1959). Since then, sialic acid and galactosamine have been added to the list of viral polysaccharide constituents (Strauss et al. 1970; McSharry and Wagner 1971).

The total carbohydrate of a virus can be estimated colorimetrically by the orcinol reaction as follows [based on Marshall and Neuberger (1972)]:

1. Dissolve 3–4 mg of dry virus in 1 ml of 0.1 N NaOH in a 10-ml glass-stoppered bylinder or test tube.
2. Add 8.5 ml of orcinol-H_2SO_4 reagent (a fresh mixture of 7.5 vol of 60 percent H_2SO_4 and 1 vol of 1.6 percent orcinol in H_2O) and mix well.
3. Place the loosely stoppered cylinder together with cylinders containing reagent and 1 ml of 0.1 N NaOH and other cylinders containing various total amounts (from 50 to 200 μg) of carbohydrates (standard solution containing equal amounts of fucose, galactose, and mannose) in a water bath at 80°C.
4. After 15 min, cool the tubes in tap water and take readings in a spectrophotometer at 505 nm.

This test will give only a crude approximation owing to small but variable contributions to the color by other constituents of the virus and by a failure to get an appropriate color yield from amino sugars. The best estimate of total nonnucleic acid carbohydrate is obtained by the summation of analyses for the individual carbohydrates.

The analysis of individual carbohydrates in viral glycoproteins and glycolipids involves a variety of methods ranging from colorimetric analyses through chromatography (paper, thin layer, column, and gas-liquid) (see Marshall and Neuberger 1972; Clamp et al. 1972). In any case, the analyses must be preceded by or include in them hydrolysis of the polysaccharides. Hydrolysis of carbohydrates has many of the features and cautions of protein hydrolysis, and these must be taken into consideration if incomplete hydrolysis is to be avoided, on one hand, and destructive hydrolysis is to be minimized, on the other. Such problems are thoroughly discussed by Marshall and Neuberger (1972).

Interference in the carbohydrate analysis can be reduced if glycopeptides are first isolated from the virus by gel electrophoresis or chromatography. Seven different carbohydrates (fucose, mannose, galactose, glucose, galactosamine, glucosamine, and neuraminic acid) were identified by gas-liquid chromatography as constituents of the nonnucleic acid carbohydrate of vesicular stomatitis virus (McSharry and Wagner 1971). Gas-liquid chromatography has attracted considerable interest for analyzing viral carbohydrates but there are technical difficulties in the method. One of them is in getting quantitative derivitization of the individual carbohydrates and another is in eliminating or reducing spurious peaks (background). Balanced against these problems are the exquisite sensitivity of the procedure (in the nanomole region) and the added specificity that can be achieved if the chromatography is coupled to a mass spectrometer or to a counter (gas-liquid radiochromatography).

3. *Function of Viral Carbohydrates*

Glucosylation of T-even phage DNAs appears to be essential for phage survival in certain bacterial strains where the glucosyl residues appear to confer resistance to degradation by nucleases. This resistance mechanism seems to be peculiar to the T-even phages and certain *E. coli* cells since other nonglucosylated phages multiply and produce infectious progeny in these bacteria.

The specific function of carbohydrates in the glycoproteins and glycolipids of enveloped viruses is not known. However, the spikes of ortho- and paramyxoviruses are glycoproteins and these are of two types, one of which constitutes the hemagglutinin of these viruses and the other a neuraminidase enzyme (see Compans and Choppin 1971). The potential importance of the carbohydrate moiety in hemagglutination is indicated by the loss of hemagglutination capacity concomitant with the cleavage of reducing sugar by a specific glycosidase enzyme (Bikel and Knight 1972). It can also be surmised because of their location in the surface of virus particles that the carbohydrates of viral envelopes play a role in the attachment and penetration of these viruses in the course of infecting cells and probably also in their exit from cells. It is not yet clear with regard to release of enveloped viruses from infected cells to what extent the viral envelope is determined by host genome and viral genome, respectively. An interesting aspect of this question is the observation that with avian tumor viruses, the glycopeptides of all viruses released from transformed cells are larger than those of viruses released from normal cells (Lai and Duesberg 1972).

Glycoproteins also are heavily involved in the immunological reactions of enveloped animal viruses. For example, antiserum to influenza hemagglutinin has potent virus-neutralizing capacity (Schild 1970), and the glycoprotein of vesicular stomatitis virus appears to be the specific antigen that induces the synthesis of and reacts with viral neutralizing antibody (Kelley et al. 1972).

E. Polyamines and Metals

In addition to protein, nucleic acid, lipid, and carbohydrate, some other substances are found in small amounts in highly purified preparations of certain viruses. Most of these minor components are probably adventitious elements. For example, many cells contain significant amounts of polyamines (Tabor et al. 1961; Cohen 1971), and these cations are strongly attracted to the phosphoryl anions of viral nucleic acids, where they remain to become a part of the mature virus particle in those cases in which low particle permeability and other relationships are favorable. Thus, putrescine, $H_2N\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}CH_2\text{-}NH_2$, and spermidine, $H_2N(CH_2)_4NH$-

$(CH_2)_3NH_2$, were found by Ames and associates (1958, 1960) in T2 and T4 phages in amounts sufficient to neutralize about half of the DNA charge. A similar situation was reported by Kay (1959) for bacteriophage 3 of *E. coli* 518.

In the case of the T2 and T4 phages, which are not normally very permeable to cations, it was shown that the putrescine is associated with the DNA inside the phage head and that this internal putrescine could not be displaced with ^{14}C-containing putrescine on the outside nor by Mg^{++} and Ca^{++}. These closely adhering polyamines are thought to be the same as Hershey's A substances (1957), which are injected along with DNA in the course of infection by T2 and T4. The lack of specificity of putrescine and spermidine was shown by growing the host cells in a medium rich in spermine, $H_2N(CH_2)_3NH(CH_2)_4NH(CH_2)_3NH_2$, a polyamine not normally present in these bacteria. The mature phages isolated from these cells were found to contain spermine rather than putrescine or spermidine (Ames and Dubin 1960).

Hence the polyamines appear to play no specific role in phages, a view which is supported by the absence of polyamines in T3, T5, and P22 phages whose permeabilities to cations presumably allow displacement of polyamines by metallic cations during isolation and purification of the phages. Furthermore, it was shown that a permeable mutant of T4 could be isolated with or without spermidine with no change in biological properties. Nevertheless, it is possible that polyamines or metallic cations may assist in the folding of DNA in the process of phage assembly. Moreover, these cations may be essential to some other stage or stages of phage biosynthesis although the mechanism of such effects is presently obscure (Cohen and Dion 1971).

Polyamines occur in the virions of herpesvirus and of influenza and Newcastle disease viruses (Gibson and Roizman 1971; Bachrach et al. 1974). Traces have also been reported in several plant viruses while amounts sufficient to neutralize about a fifth of the charges of the viral RNA have been found in turnip yellow mosaic, turnip crinkle, and broad bean mottle viruses (Ames and Dubin 1960; Johnson and Markham 1962; Beer and Kosuge 1970).

As many as 14 metallic cations have been found in plant virus preparations, some of them loosely bound to protein and others more tightly bound to RNA (Pirie 1945; Loring et al. 1958; Wacker et al. 1963; Johnson 1964). These cations can be largely removed by treatment with a chelating agent without significantly reducing infectivity. It is doubtful if the remaining few atoms of tightly bound metal are of crucial importance.

The general conclusion about both organic and inorganic cations is that they bind randomly to protein and nucleic acid in amounts dependent on the environment and relative affinities of the ions involved. Such binding, especially to the nucleic acid, may well affect the conformation and function, but specific effects have yet to be elucidated.

F. Summary: Composition of Viruses

There are many viruses in nature whose mature particles consist solely of nucleic acid and protein. There are numerous other more complex viruses that contain, in addition to nucleic acid and protein, lipid, nonnucleic acid carbohydrate, and a variety of other minor constituents. Nucleic acid and protein are properly emphasized because these constituents play a predominant role in the structure and function of viruses, although some of the minor constituents may in specific cases, as indicated in the preceding sections, be very important. Finally, nucleic acid is recognized as the one indispensable constituent of all viruses (some viruses may consist of nucleic acid alone) because it is the genetic material and is capable of inducing infection by itself.

IV.

Morphology of Viruses

The chemical constituents described in the previous chapter are found in particles of diverse size and shape in the various viruses isolable from animals, bacteria, plants, and fungi. Despite the diversity of size and shape of different viruses, the size and shape of any one virus tend to be much more uniform than do the cells of a bacterium. This uniformity is reflected in the fact that many viruses can be crystallized whereas bacteria cannot. Some examples of virus crystals are shown in Figure 30. Note that a single virus crystal contains millions of virus particles as is nicely illustrated in the electron micrograph obtained by Steere and Williams (1953) of a partially dissolved crystal of tobacco mosaic virus. Thus, although a simple virus particle may consist of hundreds of molecules of protein and one or more molecules of nucleic acid, large populations of these particles often behave as though they were just molecules, crystallization of particles being one manifestation of this characteristic (behavior of virus particles in hydrodynamic tests such as electrophoresis or sedimentation is also molecular in character).

Each virus has a characteristic size and shape. The range in size for viruses as a group is from about 20 nm in diameter for minute virus of mice to about 300 nm for a poxvirus (some elongated plant and bacterial viruses exceed this upper limit in one dimension; for example, beet yellows virus is about 10 × 1,250 nm).

Few distinctive shapes have been observed among viruses, and most viruses fall into one or another of three general groups characterized by (1) spheroidal particles (also called spherical or isometric particles); (2) elongated particles; and (3) combination particles, such as a tailed bacteriophage that may have a spheroidal head and an elongated tail. The sizes and shapes of viral particles in some distinctive groups of algal, animal, bacterial, insect, and plant viruses are given in Tables 30 to 34.

Although some attempt was made in these tables to group viruses according to recommendations of international committees concerned with virus classification and nomenclature, the purpose of the tables is not to deal with virus classification, but rather to illustrate the distribution of sizes and shapes among distinguishable classes of viruses. In order to treat viruses in groups, the dimensions assigned must necessarily encompass the

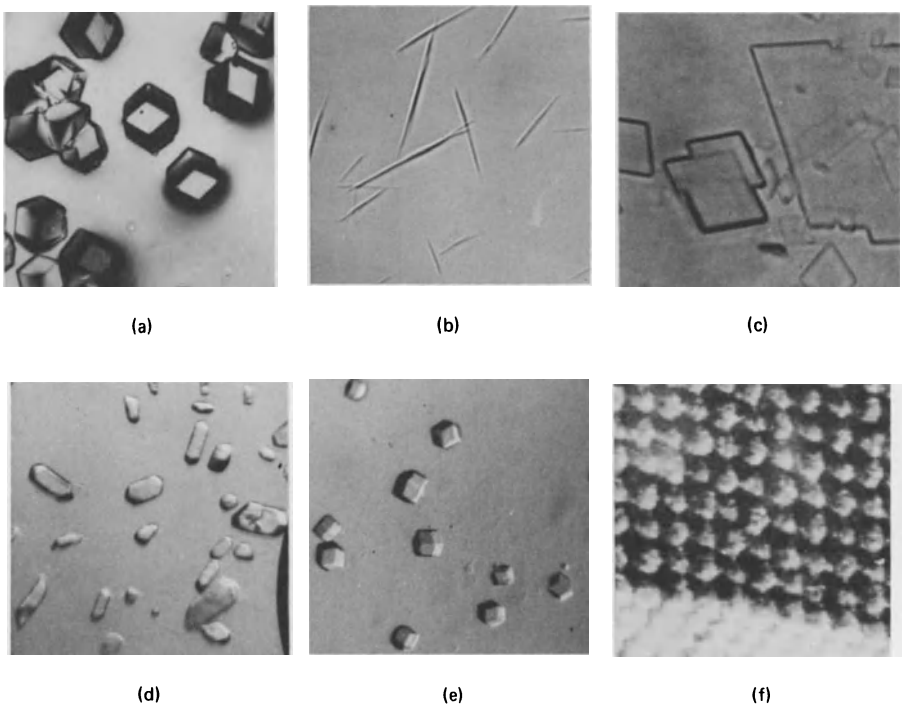


Fig. 30. Crystals of some viruses. *a*. Tomato bushy stunt virus; *b*. tobacco mosaic virus; *c*. Southern bean mosaic virus; *d*. poliovirus; *e*. polyoma virus; *f*. electron micrograph of a portion of a crystal of tobacco necrosis virus showing orderly array of virus particles. (*a* and *b*, courtesy W. M. Stanley; *d*, courtesy F. L. Schaffer; *e*, courtesy W. T. Murakami; and *f*, courtesy R. W. G. Wyckoff.)

range found in the group and thus suffer in precision for individual viruses. However, when precision in dimensions is required, it can be obtained from the references accompanying each table. Another caveat about virus dimensions is that some viruses are more plastic than others; enveloped viruses are most apt to be pleomorphic and to exhibit a range of sizes and shapes. Some of these points will be illustrated in electron micrographs of different viruses where it will also be evident that viruses occur in many sizes but in relatively few shapes.

Evidence concerning the size and shape of virus particles was obtained by indirect methods for some years prior to the common availability of the electron microscope. Some of these methods are still very useful. Thus, estimates of size can be obtained by ultrafiltration, diffusion measurements, gel chromatography, and light scattering, while indications of shape are readily obtained by flow birefringence or viscosity measurements; density

Table 30. Sizes and Shapes of Some Blue-Green Algal Viruses.[a]

Virus	Diameter or Dimensions (nm)	Shape
Anacystis, Synechococcus[b] (AS) AS-1	Head 90 Tail 23 × 244	Spheroidal head and elongated tail
Lyngbya, Plectonema, Phormidium (LPP) LPP-1, LPP-2	Head 59 Tail 15 × 20	Spheroidal head and short tail
Nostoc (N) N-1	Head 55 Tail 16 × 110	Spheroidal head and elongated tail
Synechococcus, Microcystis (SM) SM-1	88	Spheroidal with collar and possibly a very short tail

[a]Compiled from Brown 1972.

[b]These viruses are named according to the algal genera the viruses infect; hence, the names in the table are generic names of some susceptible blue-green algae. The algal viruses contain linear, double-stranded DNA. (See also Padan and Shilo 1973.)

alone, or a composite indication of size, shape , and density can be determined by various centrifugation techniques. Applications of some of these methods were described in the section on Purification of Viruses.[1]

The most versatile and direct method for determining the size and structure of virus particles is by electron microscopy. Many techniques are available that enhance the usefulness of the electron microscope beyond its ability to resolve objects down to about the 1 nm level in contrast to the approximately 200 nm resolving power of the light microscope. Some of these techniques increase contrast between virus particles and the plastic film of the microscope mount, some minimize the tendency of particles to collapse when exposed to osmotic and surface tension forces, and others limit the destructive effects of beams of electrons used to illuminate the field under examination. For descriptions of these methods and their applications, see Kay (1961), Huxley and Klug (1971), Dalton and Haguenau (1973), Williams and Fisher (1974); for reviews, see Horne (1967) and Milne (1972).

The development of electron microscopy, coupled with chemical and physical analyses, revealed various features of virus particles that might be called ultrastructural details. Many such structural components have been given names (Caspar et al. 1962; Lwoff and Tournier 1966); the commoner terms and their synonyms will be briefly presented here.

[1]Detailed descriptions can be obtained in such works as *Methods in Virology*, Vol. 2, K. Maramorosch and H. Koprowski, editors, New York: Academic Press (1967).

Table 31. Sizes and Shapes of Some Diverse Groups of Animal Viruses.[a]

Virus	Diameter or Dimensions (nm)	Shape
A. DNA-Containing Vertebrate Viruses		
Adenoviruses:	70–90	Spheroidal with projecting fibers
Avian adenoviruses		
Gallus-adeno-like (GAL)		
Chicken-embryo-lethal-orphan (CELO)		
Bovine adenoviruses		
Canine adenoviruses		
Infectious canine hepatitis virus (ICH)		
Human adenoviruses		
31 serological types		
Murine adenoviruses		
Ovine adenoviruses		
(may be same as bovine strains)		
Porcine adenoviruses		
Simian adenoviruses		
Herpesviruses:	100–150	Spheroidal with envelope
Group A		
B virus of monkeys, equine abortion, equine respiratory disease, feline rhinotracheitis, herpes simplex (types 1 and 2), infectious bovine rhinotracheitis, infectious laryngotracheitis, owl monkey herpes, marmoset herpes, squirrel monkey herpes		
Group B		
Cytomegalovirus, varicella-zoster		
Group C		
Burkitt lymphoma, herpesvirus ateles, herpesvirus saimiri,		

herpesvirus sylvilagus, Lucké frog tumor, Marek's disease of chickens		
Papovaviruses	43–53	Spheroidal
K virus of mice		
Papilloma viruses		
Bovine, canine, rabbit, human (wart)		
Polyoma of mice		
Vaccuolating viruses: rabbit, simian (SV40)		
Parvoviruses (picodnaviruses)	18–22	Spheroidal
Adeno-associated viruses (AAV)		
Hamster osteolytic viruses		
Latent rat viruses (Kilham rat virus (RV), X14, H-1, H-3)		
Minute virus of mice (MVM)		
Poxviruses	230 × 300	Brick shaped with core, lateral bodies, outer membrane with whorled surface filaments
True poxviruses		
Vaccinia-variola group		
Alastrim, cowpox, ectromelia, monkeypox, rabbitpox, vaccinia, variola (smallpox)		
Fibroma-myxoma group		
Hare fibroma, rabbit fibroma, rabbit myxoma, squirrel fibroma		
Birdpox group		
Canarypox, fowlpox, pigeonpox, turkeypox		
Sheeppox group		
Goatpox, lumpyskin disease, sheeppox		
Orf group		
Orf, bovine papular stomatitis, pseudocowpox		
Ungrouped poxviruses	150 × 200	Ovoid, with surface filaments
Molluscum contagiosum, swinepox, Yaba monkey tumor		

Table 31. Sizes and Shapes of Some Diverse Groups of Animal Viruses.[a] (cont.)

Virus	Diameter or Dimensions (nm)	Shape
B. RNA-Containing Vertebrate Viruses		
Arenaviruses Lassa, Lymphocytic choriomeningitis (LCM) Tacaribe hemorrhagic fever (several viruses)	50–150	Spheroidal with envelope
Coronaviruses Avian infectious bronchitis (IBV), several human respiratory viruses, mouse hepatitis, and rat pneumonotropic	70–120	Spheroidal with envelope
Diplornaviruses Orbiviruses (bluetongue group) African horse sickness, bluetongue, Changuinola, Chenuda, Colorado tick fever, epizootic hemorrhagic disease of deer, Eubenagee, Irituia, Palyam, simian virus SA-11, Tribec, Wad Medani	60–80	Spheroidal with inner and outer capsids
Reoviruses Avian reoviruses (5 serological types) Mammalian reoviruses (3 serological types)		

Myxoviruses	100–350	Spheroidal with envelope and projecting spikes; filamentous forms are also common
Metamyxoviruses		
pneumonia virus of mice		
respiratory syncytial (RS)		
Orthomyxoviruses	90–120	
Equine influenza, fowl plague, human influenza types A, B, and C, swine influenza		
Paramyxoviruses	120–450	
Mumps, Newcastle disease of chickens, parainfluenza (cattle, man, mice) types 1-4, simian virus 5 (SV5)		
Pseudomyxoviruses	120–300	
Canine distemper, measles, rinderpest		
Oncornaviruses	About 100	Spheroidal with envelope
Leukosis (leukemia) viruses		
Avian leukosis viruses (ALV)		
Lymphomatosis (RPL-12 and other strains), erythroblastosis (AEV), MC29, myeloblastosis (AMV), Rous-associated viruses (RAV-1, RAV-2, etc.)		
Murine leukemia viruses (MLV)		
Friend, Graffi, Gross, Kirsten, Moloney, Rauscher, etc.		
Mammary tumor viruses (MTV)		
Bittner, human (?)		
Miscellaneous oncornaviruses		
Feline, hamster, human (?), monkey, rat, reptile, etc.		
Sarcoma viruses		
Avian sarcoma viruses		
Fujinama sarcoma,		
Rous sarcoma (RSV) (several strains)		
Murine sarcoma viruses		
Harvey, Kirsten, Maloney		

Table 31. Sizes and Shapes of Some Diverse Groups of Animal Viruses.[a] (cont.)

Virus	Diameter or Dimensions (nm)	Shape
Picornaviruses	20–30	Spheroidal
Enteroviruses		
Encephalomyocarditis		
Columbia SK, encephalomyocarditis (EMC), mengo, mouse Elberfeld (ME)		
Human		
Coxsackie A, Coxsackie B, ECHO, polio		
Mouse encephalomyelitis		
Theiler's virus		
Simian enteroviruses (multiple serotypes)		
Rhinoviruses		
Human, other animals		
Unclassified picornaviruses		
Foot-and-mouth disease (FMDV), vesicular exanthema of swine (VE)		
Rhabdoviruses	75 × 130–230	Bullet-shaped or bacilliform with envelope
Bovine ephemeral fever, Chandipura, Flanders-Hart Park, Kern Canyon, Lagos, Marbur, Oregon sockeye disease, rabies, vesicular stomatitis, and others		
Togaviruses	40–60 for alpha- and flavoviruses and 100 for Bunyamuera supergroup	Spheroidal with envelope
Alphaviruses (Arbovirus Group A)		
Bebaru, Chikungunga, eastern equine encephalitis (EEE), Mayaro, Mucambo, O'nyong nyong, Pixuna, Ross River, Semiliki forest, Sindbis, Venezuelan equine encephalitis (VEE), Western equine encephalitis (WEE)		

Flavoviruses (Arbovirus Group b)
Dengue, diphasic meningoencephalitis, Japanese encephalitis, louping ill, Powassan, St. Louis encephalitis, West Nile, yellow fever
Bunyaviruses (Bunyamwera Supergroup)
Bunyamwera, California encephalitis, Inkoo
Miscellaneous togaviruses
Lactic dehydrogenase (LDH) of mice, phlebotomous fever, rubella

[a]Adapted from Melnick 1971, 1972. See also Joklik and Smith 1972, pp. 747–754.

Table 32. Sizes and Shapes of Some Bacterial Viruses.[a]

Virus	Diameter or Dimensions, nm		Shape
	Head	Tail	
A. Phages with contractile tails:			
Alcaligenes faecalis A6	90	16 × 110	Spheroidal head and rodlike tails ("spheroidal" includes oblong, octahedral, icosahedral, etc.)
Bacillus subtilis SPO1	90	30 × 210	
Escherichia coli E1	75	17 × 210	
Escherichia coli T2, T4, T6	65 × 95	25 × 110	
Lactobacillus 206	72	16 × 138	
Myxococcus xanthus MX1	75	25 × 100	
Proteus hauseri 78	61	16 × 89	
Pseudomonas aeruginosa PB1	75	20 × 140	
B. Phages with noncontractile tails:			
Escherichia coli lambda	54	10 × 150	Spheroidal head and rod-like or flexuous tail
Escherichia coli T1	50	10 × 150	
Escherichia coli T3, T7	60	10 × 15	
Escherichia coli T5	65	10 × 170	

Table 32. Sizes and Shapes of Some Bacterial Viruses.[a](cont.)

	Virus	Diameter or Dimensions, nm		Shape
	Pseudomonas Pc	65	10 × 160	
	Staphylococcus 6	40 × 92	10 × 300	
	Streptococcus 3ML	40 × 55	9 × 100	
	Typhoid 1	75	9 × 180	
	Typhoid S1 BL	50	10 × 130	
C.	Tailless phages:			
	1. With apical structures Escherichia coli α3, ØX174, ØR, S13	27		Spheroidal
	2. With apical structures but enveloped *Pseudomonas* PM2	60		Spheroidal with envelope
	3. Without apical structures[b]			Spheroidal
	Caulobacter crescentus Cb23r	22		
	Escherichia coli f2, fr, MS2, Qβ, R17	24		
	Pseudomonas aeruginosa 7s	25		
D.	Filamentous phages:			
	Escherichia coli f1, fd, M13	6 × 800		Filamentous
	Pseudomonas aeruginosa Pf	6 × 1300		
	Salmonella typhimurium If1, If2	6 × 1300		

[a]Compiled from Bradley and Kay 1960; Bradley 1971; Joklik and Smith 1972, p. 829. The phages are listed by name of host bacterium followed by designation of phage.

[b]The phages of group C3 contain RNA; all of the others are DNA phages.

Table 33. The Sizes and Shapes of Some Insect Viruses.[a]

Virus	Diameter or Dimensions, nm	Shape
A. Occluded viruses (occur in inclusion bodies):		
1. Granulosis viruses		Rodlike (occur in inclusions called granules or capsules)
Armyworm	62 × 412	
Codling moth	51 × 314	
Spruce budworm	40 × 270	
2. Polyhedrosis viruses		
Nuclear		Rodlike (occur in inclusions called polyhedral bodies)
Gypsy moth	18 × 280	
Silkworm	40 × 280	
Western oak looper	62 × 332	
Cytoplasmic		Spheroidal with surface projections (occur in inclusions called polyhedral bodies)
Monarch butterfly	67	
Silkworm (CPV)	69	
Spruce budworm	70	
3. Insect poxviruses		Brick shaped with core and lateral body (occur in inclusions called spherules)
Amsacta pox	250 × 350	
Melolontha pox	250 × 400	
4. Beetle viruses		
Melolontha (cockchaffer) spindle disease	250 × 370	Ovoid (occur in spindle-shaped or ovoid inclusions)
B. Nonoccluded viruses:		
1. Iridescent viruses Chilo, Sericesthis, and *Tipula iridescent* viruses (CIV, SIV, TIV)	150	Spheroidal

Table 33. The Sizes and Shapes of Some Insect Viruses.[a](cont.)

Virus	Diameter or Dimensions, nm	Shape
2. Miscellaneous viruses		
Acute bee paralysis	28	Spheroidal
Antherea	50	Spheroidal
Densonucleosis virus of Galleria (DNV)	20	Spheroidal
Sac brood of bees	28	Spheroidal
Sigma virus of *Drosophila*	70 × 200	Bullet-shaped with envelope

[a]Compiled from Smith 1967; Smith 1971; Bellet 1968; Bergoin and Dales 1971; Vago and Bergoin 1968; Kurstak 1972. It should be noted that there are numerous plant viruses, especially of the rhabdovirus type, that multiply in both plants and insects and are not listed here (see Knudson 1973).
The cytoplasmic polyhedrosis viruses contain double-stranded RNA and some of the miscellaneous nonoccluded viruses such as that of sac brood of bees contain RNA; all of the rest appear to contain DNA.

Table 34. Sizes and Shapes of Some Diverse Groups of Plant Viruses.[a]

Virus	Diameter or Dimensions, nm	Shape
Alfalfa (lucerne) mosaic	18 × 18; 18 × 36; 18 × 48; 18 × 58	Pleomorphic: 3 bacilliform particles and 1 spheroidal particle
Beet yellows	10 × 1250	Flexuous rods
Brome mosaic	25	Spheroidal
Also: broad bean mottle virus, cowpea chlorotic mottle		

Carnation latent Also: Cactus 2, chrysanthemum B, pea streak, potato virus M, potato virus S, red clover vein mosaic	15 × 620–700	Bent rods
Cauliflower mosaic Also: Carnation etched ring, dahlia mosaic	50	Spheroidal
Clover wound tumor Also: Maize rough dwarf, rice dwarf	70	Spheroidal
Cowpea mosaic Also: Bean pod mottle, broad bean stain, radish mosaic, red clover mosaic, true broad bean mosaic	30	Spheroidal
Cucumber mosaic Also: Cucumber yellow mosaic, tomato aspermy	30	Spheroidal
Pea enation mosaic	28	Spheroidal
Potato virus X Also: Cactus X, clover yellow mosaic, hydrangea ringspot, white clover mosaic	13 × 480–540	Flexuous rods
Potato virus Y Also: Bean common mosaic, bean yellow mosaic, beet mosaic, clover yellow vein, Columbian datura, cowpea aphid-borne mosaic, henbane mosaic, pea mosaic, potato virus A, soybean mosaic, tobacco etch, watermelon mosaic (S. Africa)	15 × 730–790	Flexuous rods

Table 34. Sizes and Shapes of Some Diverse Groups of Plant Viruses.[a](cont.)

Virus	Diameter or Dimensions, nm	Shape
Potato yellow dwarf Also: Lettuce necrotic yellows, eggplant mottled dwarf, maize mosaic, Russian winter wheat mosaic, sowthistle yellow vein	50–100 × 200–300	Bacilliform with lipid containing envelope
Prunus necrotic ringspot Also: Apple mosaic, rose mosaic	25	Spheroidal
Southern bean mosaic	30	Spheroidal
Tobacco mosaic Also: Cucumber green mottle mosaic, cucumber yellow mottle mosaic, odontoglossum ringspot, ribgrass mosaic, Sammons opuntia, sunn hemp mosaic, tomato mosaic	18 × 300	Tubular rods
Tobacco necrosis	28	Spheroidal
Tobacco rattle Also: Pea early browning	22 × 50–102 and 22 × 170–210	Tubular rods of two characteristic lengths

Tobacco ringspot Also: Arabis mosaic, grapevine fanleaf, raspberry ringspot, strawberry latent ringspot, tomato black ring, tomato ringspot	30	Spheroidal
Tomato bushy stunt Also: Artichoke mottle crinkle, carnation Italian ringspot, pelargonium leaf curl, petunia asteroid mosaic	30	Spheroidal
Tomato spotted wilt	70–80	Spheroidal
Turnip yellow mosaic Also: Andean potato latent, belladonna mottle, cacao yellow mosaic, dulcamara mottle, eggplant mosaic, ononis yellow mosaic, wild cucumber mosaic	30	Spheroidal

[a]Examples were taken mainly from Harrison et al. 1971 in which a group is defined as "a collection of viruses and/or virus strains, each of which shares with the type member all or nearly all the main characteristics of the group." All of the viruses listed contain single-stranded RNA except the clover wound tumor group, which has double-stranded RNA, and the cauliflower mosaic group has double-stranded DNA instead of RNA. The above list is an alphabetic arrangement according to type member of a group. For additional details about listed viruses see the semiannual compilations, *Descriptions of Plant Viruses*, from 1970 on, Gibbs et al. eds. These compilations are issued jointly by the Commonwealth Mycological Institute, Ferry Lane, Kew, Surrey, England, and the Association of Applied Biologists. Orders should be addressed to Central Sales Branch, Commonwealth Agricultural Bureaux, Farnham Royal, Slough SL 2 3BN, England.

The mature (structurally complete), potentially infectious virus particle is called a *virion*. Virus, or virus particle, are synonyms, although in one usage the term "virus" embraces all phases of the viral life cycle rather than just the mature virus particle. *Capsid* is a term given to the protein built around and closely associated with the viral nucleic acid, the combination of the two being called *nucleocapsid*, *nucleoprotein* (*NP*), or *core*. Synonyms for capsid are *protein coat* and *protein shell*. *Structure units* are the identical protein molecules that make up the capsid; they are also known as *protein subunits*. *Capsomers* are the capsid substructures distinguishable in the electron microscope. They may be individual protein subunits or more often represent small clusters (for example, two, five, or six) of subunits; capsomers are also called *morphologic units*. Viruses that mature at cell membranes may acquire a structure consisting of lipid, protein, and carbohydrate that surrounds and encloses the nucleocapsid, and hence is called *envelope* (*peplos* has also been suggested for this structure but has not been widely adopted). Projections from the surface of a virus particle, especially from the surface of enveloped viruses, are called *spikes* and occasionally *peplomers*. A schematic diagram of three types of virus particles showing some of these structural features is given in Figure 31.

A basic feature of virus morphology is that a virus particle is in many instances composed of numerous identical protein subunits and one or a few molecules of nucleic acid. Also, the shape of a virus particle is usually determined by the virus protein since this comprises most of the mass of the particle, and the configuration and interactions of protein subunits are essentially fixed by the amino acid sequences they possess. A combination of the data issuing from chemical, x-ray, and electron microscopic analyses with principles of symmetry from solid geometry and model building has led to the conclusion that there are two basic designs generally used in nature in the fabrication of virus particles from protein subunits: helical tubes and icosahedral shells (see Horne and Wildy 1961; Caspar and Klug 1962).

In a particle showing helical symmetry, the protein subunits are arranged in a regular helical array perpendicular to the long axis of a particle. This arrangement may result in a tubular structure such as in the tobacco mosaic virus particle (see model, Figure 18 and 7) or a flexuous strand as in the shell of elongated plant and bacterial viruses (for example, potato virus X and coliphage fd, Figure 35) or in the elongated but folded nucleoprotein components of animal viruses such as influenza, vesicular stomatitis, and Sendai viruses (Figure 34A). (Note that the helical nucleoprotein of influenza virus is enclosed in a spheroidal envelope which, though made of repeating units, cannot be readily classified in terms of symmetry.)

Icosahedral symmetry (a form of cubic symmetry) exhibited by many spheroidal virus particles requires that there be specific axes of symmetry (five-, three-, and twofold) about which the particles can be rotated to give a series of identical appearances.

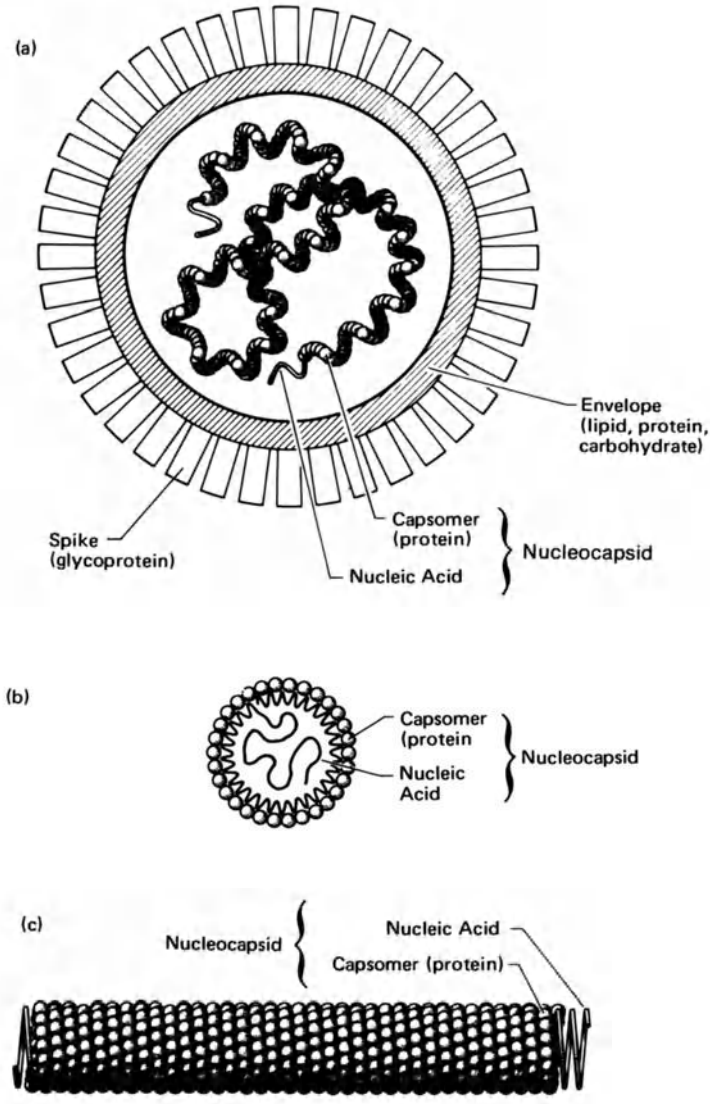


Fig. 31. Schematic diagram of three types of virions. *a*. Enveloped virion with helical nucleocapsid. *b*. Spheroidal virion. *c*. Elongated virion.

It would be an oversimplification to state that the structures of virus particles exhibit either helical symmetry or icosahedral symmetry, for some virus particles have very complex structures. Nevertheless, it is remarkable that the particle structures of many viruses can be interpreted in terms of helical and icosahedral symmetries. Some viruses exhibit both, as for ex-

ample, a tailed bacteriophage whose head may show icosahedral symmetry and the tail, helical symmetry. In terms of icosahedral symmetry it can be predicted that spheroidal viruses will have specific numbers of morphologic units. Some examples of the classes according to number of morphologic units, and some viruses possibly illustrating the classes, are given in Table 35. The numbers of protein subunits are also given in the table as a reminder that the units visualized in the electron microscope (morphologic units) usually consist of more than one protein subunit. In the Caspar and Klug concept of icosahedral viruses, the protein subunits may be thought to occur in groups of five (pentamers) and six (hexamers), as the

Table 35. Possible Numbers of Morphologic Units and Subunits in Virus Particles Having Icosahedral Symmetry.[a,b]

No. of Morphologic Units	No. of Subunits	Grouping of Subunits in Forming Morphologic Units[c]	Virus Example
12	60	12 pentamers	Coliphage ØX174
32	180	12 pentamers 20 hexamers	Broad bean mottle, cowpea chlorotic mottle, cucumber mosaic, turnip yellow mosaic
42	240	12 pentamers 30 hexamers	Arabis mosaic, tobacco ringspot
72	420	12 pentamers 60 hexamers	Human wart, polyoma, simian virus 40, Shope papilloma
90	180	90 dimers	Tomato bushy stunt, turnip crinkle
92	540	12 pentamers 80 hexamers	Reovirus, wound tumor
162	960	12 pentamers 150 hexamers	Herpes simplex, varicella
252	1,500	12 pentamers 240 hexamers	Adenovirus, infectious canine hepatitis

[a]From Knight 1974.

[b]There are classes of icosahedral particles other than those listed here, but they were omitted for lack of virus examples to illustrate them. See Caspar and Klug (1972) for a detailed discussion.

[c]These groupings of subunits are conceptual and may or may not coincide with the actual situation. For example, coliphage ØX174 seems to have four different protein components rather than 60 copies of one, and the precise numbers and morphologic arrangement of the four proteins remain to be worked out. Similarly, adenovirus has several different protein components, of which the major coat constituent, the hexon, probably consists of three polypeptides, which, moreover, are not identical.

examples in Table 35 indicate. The number of subunits per particle is 60 or some multiple of 60.

Finally, it should be noted that while the concepts of symmetry can be very important in studies of virus fine structure, molecular structures, as Caspar and Klug indicated, are not built to conform to exact mathematical concepts, but rather to satisfy the condition that the system be in a minimum energy configuration. Moreover, with modern techniques of electron microscopy, one can obtain considerable information about virus structures without any knowledge of symmetry in the mathematical sense.

In electron microscopy of viruses, contrast between particle and mount was greatly enhanced by introduction of a shadowing technique (Williams and Wyckoff 1945) in which the particles are coated obliquely with metal vapors in vacuo. This technique is tremendously useful in enhancing the contrast between virus particles and the medium on which the particles are supported, but the metal coating often obscures surface details. An exception is the Shope papilloma virus, shadowed particles of which were observed to show regular arrays of knobs (Figure 32) (Williams 1953b). This appears to represent the first direct observation of morphologic units, each of which is now thought to be composed of five or six protein subunits (see Table 35).

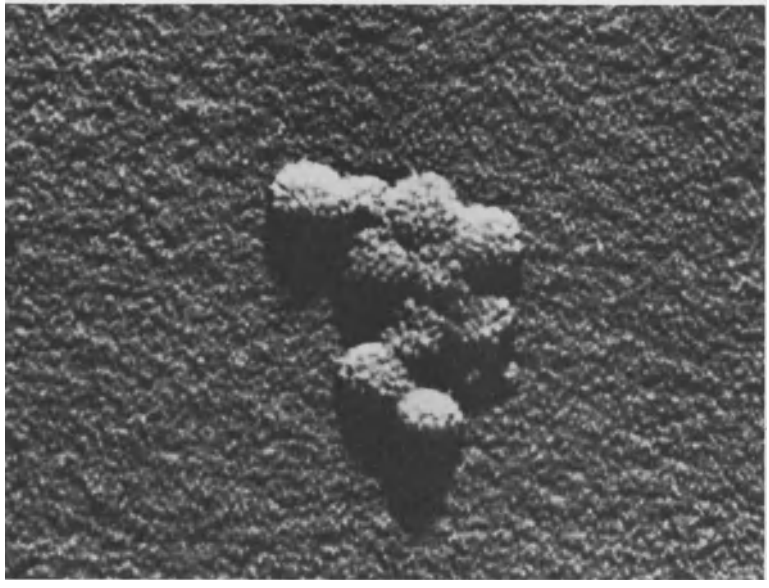


Fig. 32. Micrograph of a cluster of air-dried, uranium-shadowed particles of Shope papilloma virus showing regularly arranged surface knobs. (From Williams 1953b.)

A major advance in visualizing morphologic units as well as other structural features of virus particles occurred with Huxley's (1957) demonstration of the central hole in the TMV particle with a "negative staining" technique. This method, elaborated by Brenner and Horne (1959), was subsequently used extensively by Horne and associates (see Horne 1962) and is now universally employed. It may be briefly described as follows:

A 2 percent solution of phosphotungstic acid (PTA) is brought to neutrality or slightly above by the addition of N KOH. Equal volumes of virus (usually about 10–100 μg/ml in water or ammonium acetate) and PTA are mixed and transferred to a carbonized electron microscope grid from which much of the applied drop is removed with a small strip of filter paper. The grid is allowed to dry and then is examined in the electron microscope. Another method for applying the virus-phosphotungstate mixture is by spraying from an atomizer, giving a very fine mist. The advantage of this technique is that one can get isolated fields (spray droplets), the particles of which are more or less representative of the whole population and are contained within a single field. There are other variations of the technique, including washing of the mounts after application of the virus or virus-phosphotungstate mixture in order to remove excessive salts or small molecules, the virus generally adhering more firmly to the mount than the smaller molecules. Also, uranyl acetate or uranyl formate is sometimes substituted for phosphotungstate, especially if there is any evidence that the virus is unstable in phosphotungstate as is, for example, alfalfa mosaic virus (Gibbs et al. 1963).

With negative staining, the PTA, under the usual conditions, does not adhere specifically to the virus particles as it would in positive staining (which can be done under appropriate conditions). Rather, as the mount dries, the PTA drains down the virus particles and deposits on the particles and on the supporting mount in such a way as to reflect the topography and internal hollow regions of the particles. The micrographs presented here to illustrate the structure of different viruses were made by the negative staining technique.

A. Nonenveloped Spheroidal Viruses

Some spheroidal plant, bacterial, and animal viruses of various sizes are illustrated in Figure 33. Morphologic units are discernible in most of the particles, in some more clearly than in others. The supposed numbers of such units are indicated in Table 35. The viruses illustrated in Figure 33, as is also the case with those shown in Figures 34 to 37, are representative of dozens of other viruses (see Tables 30 to 34 for a partial listing).

Comparison of the particles shown for coliphages ØX174 and Qβ illustrates an interesting difference between these viruses. Both have spheroi-

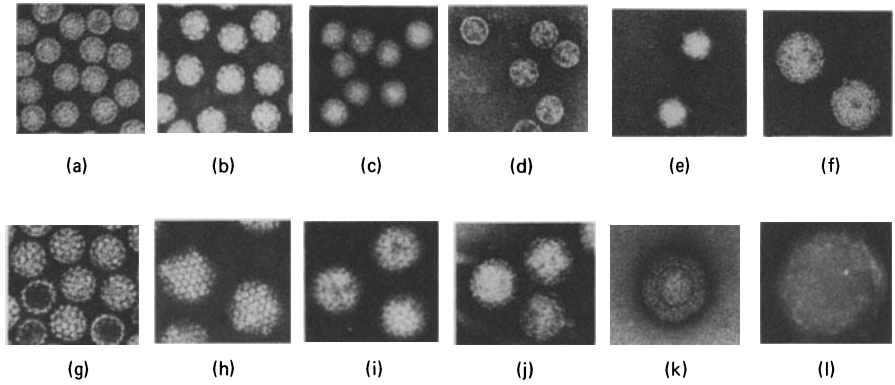


Fig. 33. Some nonenveloped spheroidal viruses. *a*. brome mosaic virus; *b*. turnip yellow mosaic virus; *c*. poliovirus; *d*. Qβ coliphage; *e*. ØX174 coliphage; *f*. cauliflower mosaic virus; *g*. Shope rabbit papilloma virus; *h*. adenovirus-5; *i*. wound tumor virus of sweet clover; *j*. cytoplasmic polyhedrosis virus of the silkworm, *k*. reovirus; *l*. *Tipula iridescent* virus. The virions in the top row are about 30 nm in diameter except for that of cauliflower mosaic, which is about 50 nm; the virions shown on the bottom row range about 50–130 nm in diameter. All mounts were prepared by the negative staining technique (see text). Note the morphologic units exhibited by some virions and especially the apical knobs on the ØX174 particles. (Courtesy R. C. Williams and H. W. Fisher.)

dal particles, but apical knobs are discernible on the ØX174 particles and not in those of Qβ. One or more of the knobs on the ØX174 particles may serve in the specific attachment of this and similar viruses to bacterial cells susceptible to infection by these phages.

Adenoviruses are among the larger viruses (about 80 nm in diameter) and consequently the faces of its icosahedral particles are more clearly evident than in smaller viruses of this shape. The particles have been studied extensively, and it is known that there are 252 morphologic units in the coat protein; these fall into two structural groups termed hexons and pentons. There are 240 hexons (each hexon consists of six protein subunits in a regular cluster) comprising most of the protein coat (capsid) of the virion. The hexons are polygonal discs about 7–8 nm in diameter with a central hole about 2.5 nm across (See Figure 33A) and each hexon is bounded by six other morphologic units. The 12 pentons are situated at the 12 vertices of the icosahedron and each is bounded by five morphologic units. The pentons serve as base structures to which fibers, called penton fibers, are attached. Each penton fiber is about 2 × 20 nm and terminates in a spherical knob about 4 nm in diameter. These structures are often invisible in the electron dense PTA medium employed in negative staining, but in areas where the PTA matrix is less dense they can be discerned as shown with one particle in Figure 33A. The penton fibers are important in the serologic and hemagglutinating activities of adenoviruses and may also

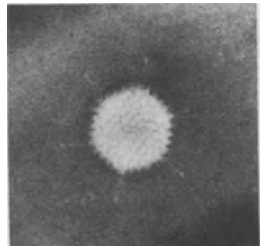


Fig. 33A. An adenovirus virion showing some hexon and penton units (Courtesy R. C. Williams and H. W. Fisher.)

serve as attachment organs in initiating the process of infection (Horne 1973).

It is not uncommon to find particles in preparations of various viruses that, although otherwise closely resembling intact particles in size and shape, are lacking in nucleic acid. Such empty particles exhibit dark centers on electron micrographs, presumably reflecting the ability of PTA to flow readily through empty viral shells and puddle beneath the particles in larger amounts than under complete particles. This is illustrated in the micrograph of Shope papilloma virus in Figure 33. It should be noted in this connection that phosphotungstate may cause a proportion of initially full particles to leak out their nucleic acid (Milne 1972), since a much smaller percentage of empty particles is observed with sensitive viruses when uranyl acetate is employed as the negative stain.

The particles of diplorna viruses such as reovirus are distinctive in containing segmented, double-stranded RNA, as well as for having two protein shells, an outer and an inner one. The morphologic units of both shells are arranged according to icosahedral symmetry. The outer shell appears as a ring in the micrograph of reovirus shown in Figure 33. The outer shell can be digested away with chymotrypsin to leave the inner nucleocapsid, or "core." However, it is not yet clear whether other diplornaviruses have double capsids. The particles of wound tumor virus of sweet clover (WTV) (Figure 33) and of silkworm cytoplasmic polyhedrosis virus (CPV) approximate the size of reovirus cores rather than whole particles and are also similar to cores in possessing RNA transcriptase activity (Lewandowski and Traynor 1972). Of course it is possible that the outer capsids of WTV and of CPV are more readily lost in isolating these viruses than is that of reovirus. This is especially a possibility with CPV, which is usually extracted from polyhedral bodies at rather high pH.

The particles of the iridescent insect viruses (the term "iridescent" comes from the fact that diseased tissues as well as gelatinous pellets of

purified virus obtained by centrifugation are iridescent when examined by reflected white light) are the largest presently known nonenveloped viruses; they have a diameter of about 150 nm and are clearly icosahedrons (Williams and Smith 1958). Negatively stained particles, such as those of *Tipula iridescent* virus shown in Figure 33, exhibit a hexagonal outline on micrographs and the protein coat appears as a membranous one- or two-layer structure.

A general point can be made here concerning the relationship between protein and nucleic acid in viruses. There is no evidence for covalent linkage of these substances in any type of virus; nevertheless, the secondary attractions between protein and nucleic acid tend to result in specific configurations. With respect to spheroidal viruses, the nucleic acid is not just randomly packed in a protein shell. For example, x-ray analyses made on turnip yellow mosaic virus (Klug et al. 1966) indicate a regular interlacing of the nucleic acid with the protein subunits far enough below the surface of the particle to protect the nucleic acid from outside degradative agents. The crude sketch of Figure 31 is intended to suggest this relationship as opposed to the simple bag-of-nucleic-acid concept. A similar structure has been deduced for broad bean mottle virus (Finch and Klug 1967). However, specific details of protein-nucleic acid association for spheroidal viruses, as well as for most viruses, are yet to be elucidated.

B. Large, Enveloped Spheroidal and Elongated Viruses

Numerous, large (70 nm in diameter or greater) animal and plant viruses share the feature of maturing at cell membranes (nuclear, vesicular, cytoplasmic) through which they bud, acquiring an envelope structure in the process. The envelope is composed of both host and viral components, the protein tending to be virus specific, while the lipid and perhaps carbohydrate may be characteristic of the host membrane. Quite often, discernible protuberances called spikes (see Figure 31) are apparent in negatively stained preparations of virions. In cases where they have been most thoroughly studied (ortho- and paramyxoviruses), the spikes are rod-shaped structures about 4–5 × 8–14 nm and appear to be glycoproteins (Compans and Choppin 1973).

Two viruses whose morphologies exemplify numerous spheroidal enveloped viruses are illustrated in Figure 34. They are influenza (a myxovirus) and Rous sarcoma virus (an oncornavirus); some other viruses belonging in these groups are listed in Table 31. Such enveloped viruses tend to be plastic and thus exhibit pleomorphism, which is illustrated in Figure 34 with the influenza virions shown there. While a myxovirus tends to be spheroidal in shape, its nucleoprotein constituent is usually an elongated

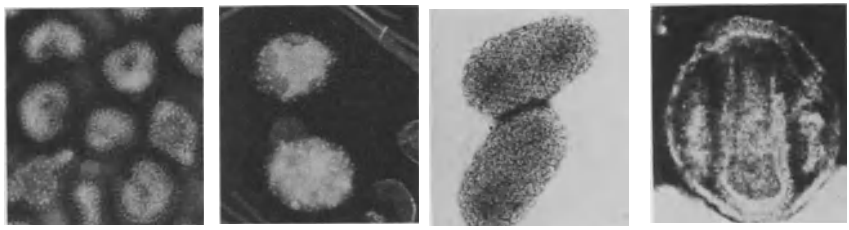


Fig. 34. Three large enveloped viruses and a poxvirus. *a*. influenza virus; *b*. Rous sarcoma virus. *c*. sowthistle yellow vein virus; *d*. vaccinia virus (a poxvirus). Note the pleomorphism and peripheral spikes of the influenza virions. The vaccinia virus particle has been partly stripped with detergent in order to reveal core and lateral bodies. (*a* and *b*, courtesy R. C. Williams and H. W. Fisher; *c*, courtesy D. Peters and *d*, courtesy K. B. Easterbrook.)

structure with helical symmetry that exists in a folded or coiled state within the envelope. A segment of nucleoprotein (nucleocapsid) released from the paramyxovirus, Sendai virus, is illustrated in Figure 34A. This same sort of structure has also been associated with the nucleocapsids of rhabdoviruses.

In both ortho- and paramyxoviruses the nucleocapsid is composed of a single polypeptide species associated with single-stranded RNA in an elongated helical structure similar to that for Sendai nucleocapsid in Figure 34A. However, a basic difference is that the nucleocapsid of paramyxoviruses appears to exist in a single helical structure, whereas that of the orthomyxoviruses may occur in or readily dissociate into several segments (Compans and Choppin 1973). There are hints that such a segmented nucleocapsid may also occur in oncornaviruses (Tooze 1973).

Among the enveloped viruses, herpesviruses are unique in having an icosahedral rather than a helical nucleocapsid. This group of large spheroidal, enveloped viruses has many members, including some with oncogenic properties (see Table 31) (Roizman and Spear 1971).

Another type of large enveloped virus is represented by the group called rhabdoviruses, which has representatives among both animal and plant viruses. Vesicular stomatitis virus, which has bullet-shaped particles (rod with one end rounded and the other planar), is representative of numerous other animal viruses (Table 31) (Hummeler 1971). Such bullet-shaped particles have been observed also with plant viruses, but a bacilliform shape (rod with both ends rounded) seems more characteristic of undegraded particles of these viruses as illustrated by the virion of sowthistle yellow vein virus shown in Figure 34 (see also Knudson 1973). Some other bacilliform viruses in the plant series are listed in Table 34 under potato yellow dwarf, the morphological prototype of this group.

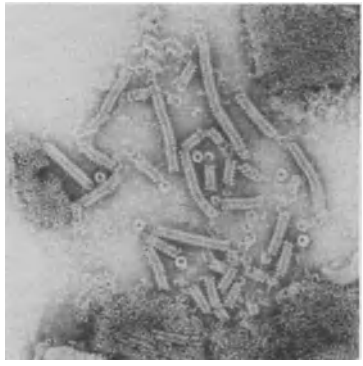


Fig. 34A. A segment of the helical nucleocapsid of Sendai virus. Compare with Fig. 35*c* and 35*d*. (Courtesy R. C. Williams and H. W. Fisher.)

C. Brick-Shaped Viruses

Poxviruses are the largest and most complex of the animal viruses; their virions are usually described as brick- or loaf-shaped. Whether isolated from insects, birds, or mammals (see Tables 31 and 33) (Dales 1973), a basic structural pattern is observed in the virions; they have a highly convoluted, tubular, lipoprotein outer membrane, an internal protein-nucleoprotein core (sometimes called nucleoid), and proteinaceous lateral bodies. These latter features are illustrated by the micrograph of vaccinia virus in Figure 34. In addition to the double-stranded DNA and associated protein, the cores of vaccinia virus enclose four enzymes: a RNA polymerase (transcriptase), a nucleotide phosphohydrolase, and two deoxyribonucleases (DNases)—one an exonuclease and the other an endonuclease. While the function of the lateral bodies is not definitely known, they may serve as inhibitors of the viral DNases since both DNases show elevated activities if the lateral bodies are removed from cores by treatment with a proteolytic enzyme (Dales 1973). Thus the lateral bodies could restrain the activity of the DNases in the vaccinia virions but upon removal during the course of infection might release them to attack host cell DNA.

D. Elongated Viruses

The two basic elongated structures of virions observed thus far are tubular and filamentous particles. They have been noted for several bacterial and plant viruses (Table 32 and 34). Two examples of each type of structure are shown in Figure 35.

Tobacco mosaic virus is the best known and most thoroughly studied rod-shaped virus. The structure of TMV virions was rather well understood by the time negative staining was developed, so this technique only served to confirm the morphology already established by chemical and x-ray studies. Since the approach used for TMV is a classical one for deducing structure of rodlike particles, it will be briefly sketched here.

There was evidence that TMV protein was a single species that occurred in about 2,000 identical subunits (molecular weight about 18,000) per virion of 40×10^6 daltons (Harris and Knight 1955). It was further known that the RNA of TMV was a single-stranded molecule with a molecular weight of about 2×10^6 and about 3,300 nm long, which ran the length of the TMV rod (Hart 1958; Gierer 1957). Important information missing at this time were the arrangement of the protein subunits and the spatial relationship that protein and nucleic acid took with respect to one another. This was supplied by study of the low-angle x-ray scattering patterns yielded by concentrated gels of purified TMV (Watson 1954; Franklin et al. 1957, 1959; Caspar 1956). The x-ray data indicated that the protein subunits of the virus are arranged in a helical array about the long axis of the virus rod; that there is a central hole about 4 nm in diameter so that the rod is actually a tube; that there are regions of high and low density in the particle at specific radii; and, by comparison of radial density distributions of complete and nucleic acid-free particles, that the nucleic acid is not in the center of the tube but is intermeshed with the protein subunits at a radius of about 4 nm. Some of these points are evident from the radial density distribution diagrams shown in Figure 36. As indicated in the figure, density distribution curves similar to that of TMV were also obtained with three strains of this virus and for cucumber virus 4; it will be noted that the curves all show maxima at the same radii and differ mainly in

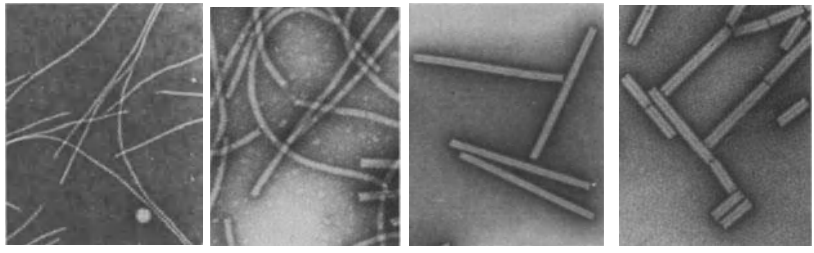


Fig. 35. Some elongated viruses. *a*. coliphage fd; *b*. potato virus X; *c*. tobacco mosaic virus; *d*. tobacco rattle virus. The particles of phage fd and of potato virus X are too long to be shown in their entirety at the magnification used here. (Courtesy R. C. Williams and H. W. Fisher.)

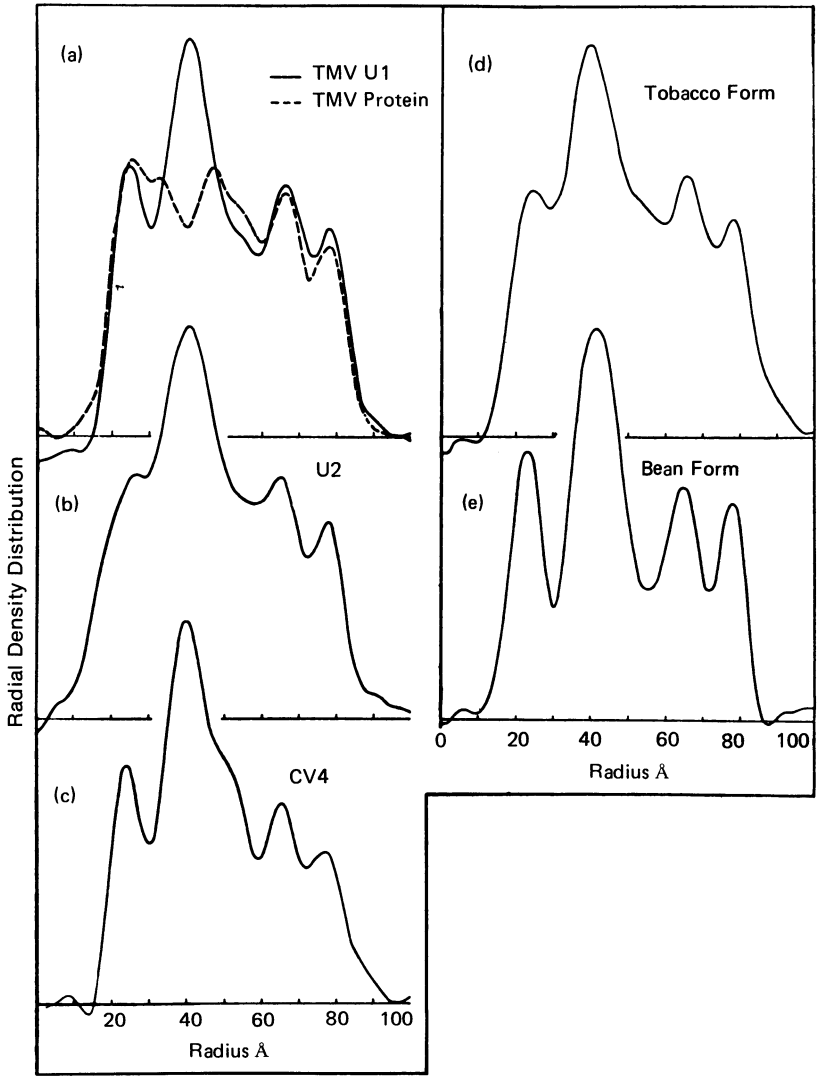


Fig. 36. The cylindrically averaged, radial, electron density distributions of tobacco mosaic virus, some of its strains, cucumber virus 4 (CV4) and TMV protein. The curves show the difference between the electron density of the particles and that of water plotted as a function of radial distance from the particle axis. TMV U1 is common TMV (also called vulgare and wild type) and U2 is a mild strain of TMV (Siegel and Wildman 1954). The strains represented in (*d*) and (*e*) originated in Nigerian cowpea (Bawden 1958). (From Klug and Caspar 1960; see also Caspar 1956; Franklin et al. 1957.)

quantitative respects, which probably represent slight differences in packing of material.

Putting all the evidence together, a model of the TMV particle can be constructed illustrating the helical arrangement of protein subunits in the TMV shell and the manner in which the RNA strand intermeshes with the protein subunits and assumes the helical configuration of the subunits (Figures 7 and 18).

In Figure 35 the central hole is evident in the virions of TMV and of tobacco rattle virus. Cross striations also delineate the helical array of subunits in all of the elongated virions. It will be noted that the filamentous viruses exhibit flexuous shapes rather than the straight form shown by the elongated viruses with greater cross-sectional diameters.

As with other viruses, including the isometric ones, the protein subunits of elongated viruses are associated with the nucleic acid by noncovalent bonds. However, in some cases the stability of this structure is very great; for example TMV has been reported to retain infectivity in extracts at room temperature for 50 years (Silber and Burk 1965).

E. Tailed Viruses

Some bacterial viruses are characterized by spheroidal particles, some by filamentous particles, and many are combinations in which head and tail structures are evident. In the latter case, head capsid may exhibit icosahedral symmetry and the tail helical symmetry. Among the tailless phages there is at least one known, *Pseudomonas* PM2, which has a lipoprotein envelope (Espejo and Canelo 1968; Silbert et al. 1969) that appears to fit closely around an icosahedral capsid. This phage is unusual also in being the only tailless phage possessing double-stranded DNA (which happens to be circular).

The head sizes of different tailed phages vary considerably and the shape ranges from almost spherical to oblong. The head houses the nucleic acid (apparently always double-stranded DNA), while the tail serves as an attachment organ in the initial step of infection and a tube through which the DNA travels in a subsequent step (penetration). Some tails are short, some long, some straight, and some curved; they vary tremendously in complexity, especially with regard to possession or not of accessory structures such as collars, base plates, spikes, tail fibers, and so on. Many of these features are illustrated in Figure 37 and characterize numerous phages, some of which are listed in Table 32.

Tailed viruses have also been observed as infectious agents of blue-green algae (Table 30); two of these are illustrated in Figure 37. The N-1 algal virus (Adolph and Haselkorn 1971), as can be seen in the figure, resembles long-tailed bacteriophages, especially those with contractile sheathed tails. The SM-1 algal virus (MacKenzie and Haselkorn 1972)

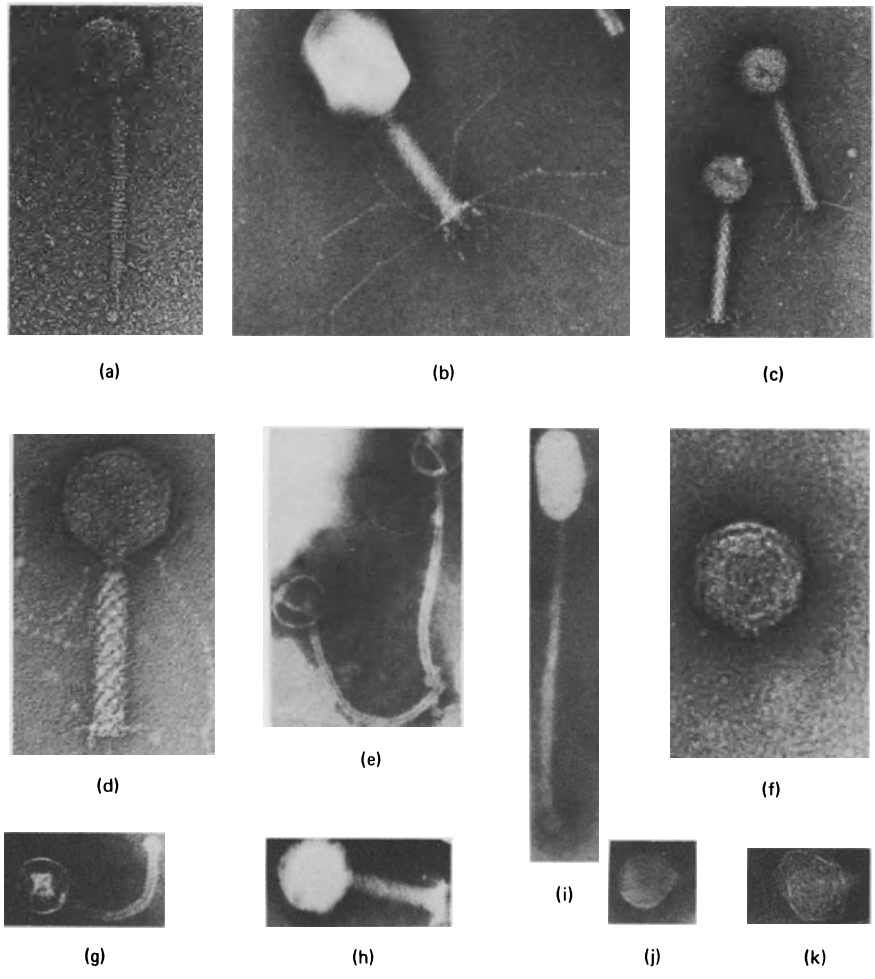


Fig. 37. Some tailed bacteriophages and algal phages. *a*. coliphage lambda; *b*. coliphage T4; *c*. coliphage P2; *d*. N-1 algal virus (from *Nostoc muscorum*); *e*. *staphylococcus* phage 77; *f*. SM-1 algal virus; *g*. *pseudomonas* phage Pc; *h*. typhoid phage Vi 1; *i*. *staphylococcus* phage 6; *j*. a *brucella* phage; *k*. coliphage T7. Mounts were all prepared for electron microscopy by the negative staining technique. (*a, b, c,* and *k,* courtesy R. C. Williams and H. W. Fisher; *d* and *f,* courtesy R. Haselkorn; and the rest, courtesy D. E. Bradley and D. Kay.)

resembles the short-tailed phages; it has an icosahedral head capsid from which there protrudes a collar and a short appendage that could be a tail.

F. Encapsulated Viruses

There are two morphologically different classes of insect viruses that may be called occluded or nonoccluded, depending on whether they typically appear in their mature form in special inclusion bodies or not (Table 33). The nonoccluded virus of *Tipula paludosa* (the crane fly or daddy longlegs), called *Tipula iridescent* virus, is illustrated in a micrograph in Figure 33. However, most insect viruses appear to occur in their mature form in characteristic inclusion bodies. These inclusion bodies are generally crystalline protein packages that contain one or more virus particles. Some of these packages are called polyhedral bodies, and are found characteristically in either nuclei or cytoplasm of infected cells; the diseases associated with them are correspondingly termed nuclear polyhedroses and cytoplasmic polyhedroses. The occluded virions of nuclear polyhedroses are generally rod-shaped, while those of the cytoplasmic polyhedroses are spheroidal and have icosahedral capsids. Hundreds of virus particles are occluded in the crystalline protein matrix of each polyhedral body whether nuclear or cytoplasmic; they can be released by treatment with dilute alkali. For example, the cytoplasmic polyhedrosis virus of the silkworm is released from polyhedra by holding the polyhedral bodies at 25° in 0.1 M NaCl and 0.05 M Na_2CO_3 at pH 10.6 for 1 hr (Lewandowski et al. 1969).

In the insect diseases called granuloses, the inclusion bodies are called granules or, more frequently, capsules. Some distinctions between capsules and polyhedral bodies are

1. Shape of the inclusion bodies: polyhedral bodies occur in a variety of shapes depending on the polyhedrosis involved and have been described as dodecahedral, tetrahedral, rectangular, hexagonal, and crescent-shaped; capsules are usually described as ovoid or egg-shaped in outline although some cubic capsules have been reported.
2. Size: the polyhedral bodies vary in size both in the same and in different polyhedroses but in general they are much larger than capsules and range from 500 to 15,000 nm in diameter, whereas the range of sizes of capsules is more of the order 119 to 350 nm wide and 300–511 nm long.
3. Number of virus particles occluded: hundreds or thousands of virions may be found in polyhedral bodies but on the average only one virion occurs in a capsule.

Thin sections can be made of polyhedral viruses and capsules which upon electron microscopy reveal the dispersion of virus particles (Figure 38). Two concentric membranes can be observed surrounding each virus particle in nuclear polyhedral bodies and capsules but not in cytoplasmic polyhedral bodies. The membranes, when present, are termed inner or intimate membrane (next to the virion) and outer membrane. Their precise functional relationship to the virions is not yet clear. A cross section of a nuclear polyhedral body showing the occluded cabbage looper virus particles and a similar section of a capsule showing a meal moth virus are shown in Figure 38.

As indicated in Table 33 there are at least two other types of occluded insect viruses. One of them occurs in inclusion bodies called spherules and the occluded virus appears to be a poxvirus (Bergoin and Dales 1971); the other is a beetle virus found in peculiar spindle-shaped or ovoid inclusions (Vago and Bergoin 1968).

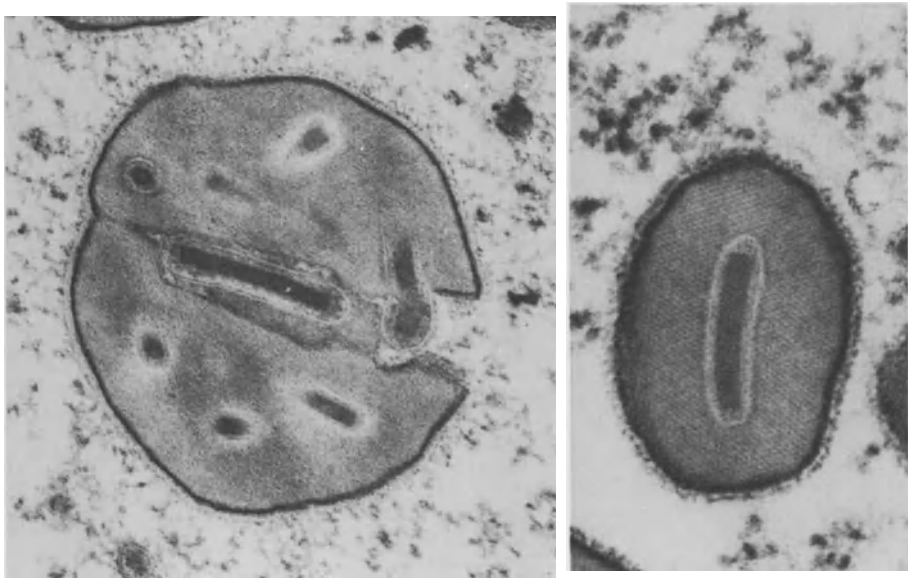


Fig. 38. Two types of occluded insect viruses. *a*. Thin section of a polyhedral body from the nuclear polyhedrosis of the cabbage looper (*Trichoplusia ni*). Bits of the randomly oriented viral rods are apparent in the section with a complete rod discernible in the center of the section. *b*. Thin section from a capsule of the granulosis of the meal moth (*Plodia interpunctella*) showing the single virus particle embedded in a crystalline capsule. (*a*, courtesy M. D. Summers; *b*, courtesy H. J. Arnott and K. M. Smith.)

V.

Action of Chemical and Physical Agents on Viruses

The reaction of viruses with chemical and physical agents seemed rather complex and mysterious some years ago when the structure of viruses and the basic features of the process of infection were poorly understood. Now, the details may still be intricate and incompletely defined, but the main facts relating chemical and physical treatments of viruses to the biological activity of viruses are simple and clear:

In order for a given infectious virus particle to remain fully infectious, the chemical structure of its nucleic acid must not be irreversibly harmed and the nucleic acid must be capable of release from the virion in a form that can react normally with transcription-translation apparatus (enzymes, attachment factors, and so on). A prediction of this formulation is that it should be possible to inactivate viruses in two general ways: (1) by changes in the nucleic acid that render it partly or wholly nonfunctional in the central dogma scheme (self-replication, transcription, translation) or (2) by alteration of the protein coat or other structures of the virion (for example, tail fibers of a phage, RNA polymerase of a poxvirus) in such a way as to prevent delivery of the viral nucleic acid into a functional area of the cell. Both types of inactivation have been detected and both can be caused by heat, radiation, and chemicals.

In addition to the two general types of inactivation that chemical and physical treatment of viruses can produce, they can also produce noninactivating, heritable changes in the nucleic acid. This is called mutation, which will be considered below. Actually, moderate treatments with some agents cause mutation while harsher treatments cause inactivation; in practice, a combination of the two effects is often observed.

A. Inactivation of Viruses

The primary characteristic that makes a virus a virus is its infectivity, that is, its ability to cause the production of progeny like itself. Therefore, the term "inactivation of viruses" is used here to mean the abolishment of infectivity even though it is possible for chemical and physical treatments

to affect other characteristics of viruses instead of, or as well as, infectivity. Conversely, it is often possible to eliminate infectivity without destruction of antigenic or serologic reactivity (a fact used in the production of one type of vaccine), and virus particles that have lost the capacity to reproduce fully sometimes still can induce virus inhibitory substances (interferons), cell fusion, enzyme production, and oncogenic transformation of cells (Potash 1968; Kleinschmidt 1972; Watkins 1971a, 1971b; Mathews 1971; Kajioka et al. 1964; Rubin 1965). These examples are explicable on the basis that many of the properties of viruses are expressed by their protein coats and/or envelopes, and that partial transcription (or translation) of viral genomes is known to occur. Thus the nucleic acid of a virion can be altered to the point that production of complete virus is blocked but several viral functions can still be performed.

When inactivation of viruses is reduced to the two simple terms outlined above—alteration of the exterior of the virion or of the nucleic acid—much of the earlier mystery of inactivation is dispelled, and specific explanations for the effect of heat, radiations, chemicals, and so on can be sought in a logical, systematic way. There are practical consequences of this view too. For example, if one is interested in producing a vaccine constituted from an inactivated virulent strain of virus, it is clear that selection of an agent that will attack mainly the nucleic acid is better than an agent that inactivates by altering the viral attachment sites. First, the type of agent that mainly attacks nucleic acid preserves more of the antigenic structure of the exterior of the virus and hence should elicit a better immune response. But, equally important, the nucleic acid is inactivated in this case, whereas when inactivation ensues from altering virus surface structure, the nucleic acid may be intact and could by some fortuitous circumstances (for example, local changes in pH or salt concentration, presence of a latent helper virus) be released from its shell to initiate a devastating infection.

To carry the example further, even use of an agent such as formaldehyde, which can get through the virion superstructure and react efficiently with the nucleic acid, does not guarantee inactivation of the virus to a safe level. Formaldehyde reacts with proteins as well as with nucleic acids and prolonged reaction causes cross-linking between various groups of the protein (so-called tanning or membrane forming effect) (Fraenkel-Conrat and Olcott 1948). Consequently, it can become more difficult for formaldehyde to penetrate the viral shell, and Gard (1957) demonstrated with poliovirus that as inactivation with formaldehyde proceeds, the residual infectivity of the virus becomes progressively more resistant to inactivation. Another point in respect to the inactivation of viruses is that there are some ways of inactivating viruses that are reversible in the cell, such as inactivation with moderate doses of ultraviolet light (see section on Inactivation of Viruses by Radiations).

A uniformity in the kinetics of inactivation of all types of viruses was recognized some time before it was clear that there were two general modes of inactivation of viruses (Luria and Darnell 1967, Chap. 7). Thus, regardless of whether the inactivating agent is chemical or physical, loss of infectivity often follows an exponential decay law based on the simple first-order reaction formula

$$V/V_0 = e^{-kt}$$

where V is infectivity at time t, V_0 is initial infectivity, and k is the rate constant. If the logarithm of infectious titer is plotted against time, a straight line is observed (Figure 39). This means that a constant fraction of the virions undergoes a change, causing loss of infectivity in each unit of time, and that one such independent change is sufficient to inactivate a virion (one-hit theory). In viral structural terms, infectivity can be lost, for example, by a single change in a viral protein involved in the initiation (for example, attachment) step of infection, by a single change in the RNA

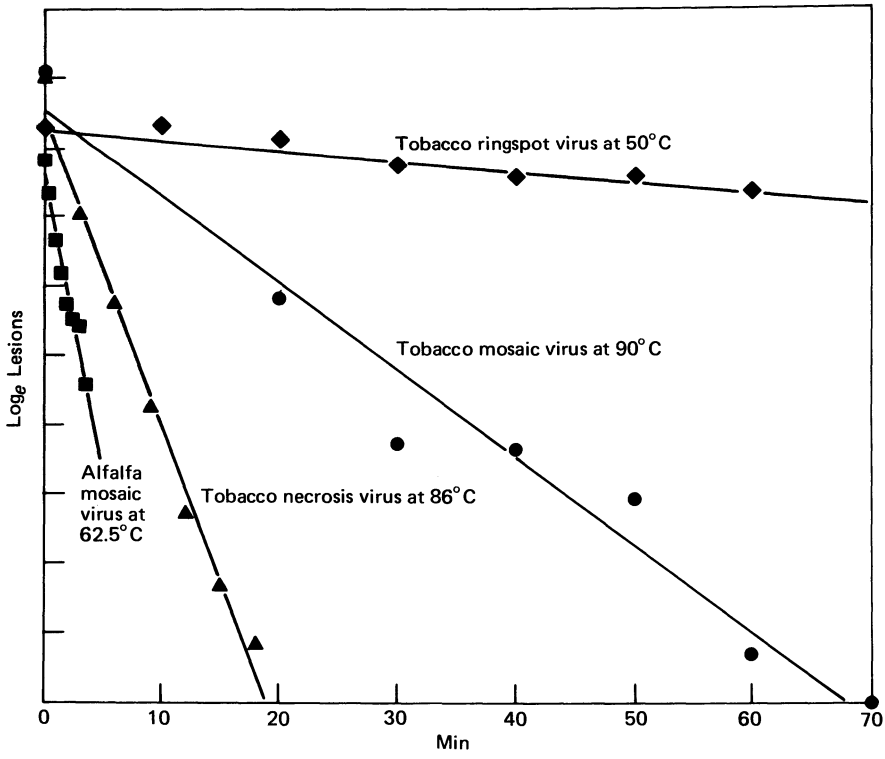


Fig. 39. Thermal inactivation rate of some plant viruses. (Adapted from Price 1940.)

polymerase of a virion containing such an enzyme, or a single change in the viral nucleic acid. The same type of kinetics applies to the production of mutants if the rule is restricted to events affecting the viral nucleic acid.

1. Inactivation of Viruses by Heat

The infectious quality of most viruses persists quite well in the cold and especially well below freezing such as at the temperature of dry ice (−70°C) or of liquid nitrogen (−196°C). However, there is a great variation in the lability of different viruses at elevated temperatures. At one extreme the infectious half-life of enveloped viruses such as the myxoviruses and RNA tumor viruses may be only an hour at 37°, while at the other extreme some plant viruses such as tobacco mosaic virus are known to have maintained some infectivity stored in plant sap at about 20°–25°C for 50 years and can stand 80°–90° for 10 min with only a moderate loss of infectivity (Silber and Burk 1965; Price 1940).

Much of the loss of infectivity of viruses at temperatures between 25° and 70°C can doubtless be attributed to changes in protein components of the viruses since proteins in general are more readily denatured by heat than are nucleic acids. Furthermore, nucleic acids, located as they always are in the interior of virions, receive substantial protection from thermal and other degradative assaults. Above 70°C, strand separation occurs with double-stranded nucleic acid and finally random breaks occur in the sugar-phosphate backbone of all types of nucleic acids. Thus, at high temperatures both protein and nucleic acid can be seriously and often irreversibly damaged. In addition, the lipid-containing envelopes of some viruses are doubtless degraded at moderate to high temperatures.

In the context of the process of infection, heat-induced changes in configuration of viral surface protein (including glycoprotein and lipoprotein) could be expected to result in one or more of the following consequences: (1) prevent the specific attachment of virions to cell receptor sites (and thus block the initial step of infection); (2) inactivate virion-associated enzymes needed for virus replication; (3) hinder removal of coat protein and release of viral nucleic acid.

It should be noted that heat inactivation is modulated by other environmental conditions such as the presence of extraneous proteins and divalent cations such as Mg^{++} and Ca^{++}. Conversely, the presence of protease and nuclease enzymes can be expected to increase inactivation observed with heat, especially at moderate temperatures.

2. Inactivation of Viruses by Radiations

In their effect on matter, electromagnetic radiations can be considered to fall into two classes: ionizing and nonionizing. Gamma rays and x-rays

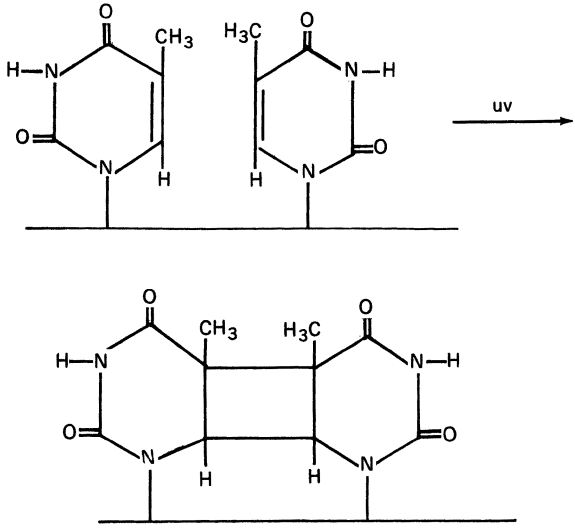
are common ionizing radiations used in virology, while ultraviolet light is the predominant nonionizing radiation employed. Ionizing radiations provide much higher energy than nonionizing radiations; for example, 0.1 Å x-rays have about 25,000 times the quantum energy of ultraviolet light at 2,537 Å. However, ionizing radiations show little selectivity, their effect on a virus being governed by little more than the atomic density encountered in various portions of the virion. In contrast, the purine and pyrimidine rings of nucleic acids strongly absorb nonionizing ultraviolet light (proteins absorb this radiation too, but much more weakly and mainly in proportion to their aromatic amino acid content). The main inactivating effect of either type of radiation is assumed to be in the viral nucleic acid. Reviews of techniques for using ionizing and nonionizing radiations in virology have been made by Ginoza (1968) and Kleczkowski (1968).

Both types of radiations inactivate viruses by direct effects, and ionizing radiations may also inactivate by indirect effects stemming from the production in aqueous media of hydrogen and hydroxyl-free radicals and of peroxides (Luria and Exner 1941; Watson 1950). An example of the indirect effect is that phages can be inactivated by free radicals, the mechanism appearing to be a radical attack on the phage tail causing a premature release of DNA from the phage head (Dewey 1972). The direct inactivating action of an ionizing radiation such as x-rays involves rupture of covalent bonds in protein or, more likely, with moderate doses, in nucleic acid (Freifelder 1965, 1966; Summers and Szybalski 1967; Lauffer et al. 1956). In the latter instance, strand scission is more serious with single-stranded nucleic acid than with double-stranded nucleic acid since rupture of complementary strands in the same vicinity is required in order to disrupt double helical structures, whereas a break anywhere severs single-stranded nucleic acid. This is reflected in the approximately tenfold greater efficiency of x-rays in inactivating viruses containing single-stranded nucleic acid as opposed to those containing double-stranded nucleic acid.

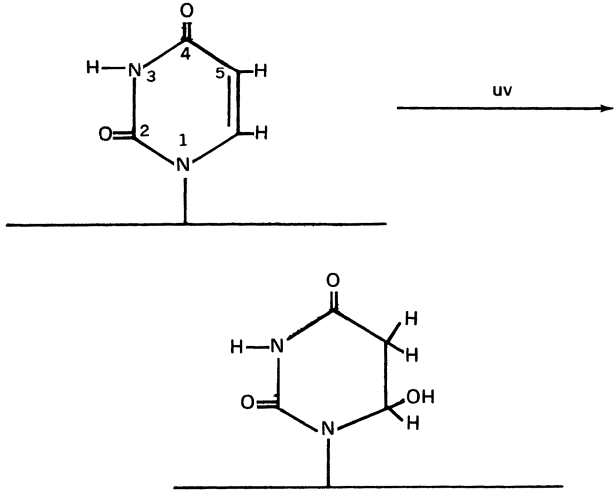
In contrast to ionizing radiations, ultraviolet light can inactivate viruses without breaking polynucleotide chains (chain breaks, which also are inactivating, can occur at doses of 10^4 ergs/mm^2 or higher). Inactivation by moderate doses of ultraviolet light appears to be attributable to one or more of three observed effects of ultraviolet light on pyrimidines in polynucleotide chains (McLaren and Shugar 1964): (1) covalent bonds may be formed between adjacent thymine residues in DNA or between uracil residues in RNA to form so-called thymine dimers or uracil dimers, respectively; (2) hydration may occur at the C5-C6 double bond of pyrimidines to form 5-hydro-6-hydroxy derivatives (these are thought to be especially important in the inactivation of RNA-containing viruses); and (3) some cross-linking may occur, probably involving pyrimidines, between complementary chains of double-stranded nucleic acids.

Thymine dimer formation may be shown as follows, the horizontal

straight line representing the sugar-phosphate backbone of a strand of nucleic acid containing adjacent thymine residues:



The formation of a uracil hydrate may be represented as follows:



It has been shown that dimers can be removed from bacterial DNA by two types of enzymatic mechanisms, and it is supposed that these may also function in plant and animal cells since reversal of ultraviolet radiation

damage has been observed under conditions analogous to those used to accomplish reversal in bacteria. One reactivation system is photoreactivation (Kelner 1949; Dulbecco 1950; Setlow 1968) in which light at 3,500–4,500 Å in concert with an enzyme is able to split thymine dimers (Setlow 1968; Howard-Flanders 1973). Photoreactivation by such a light-activated enzyme system may not occur with some RNA-containing viruses such as tobacco mosaic virus, apparently because of interactions between protein and nucleic acid; however, infectious RNA from TMV can be photoreactivated following treatment with ultraviolet light (Bawden and Kleczkowski 1959; Rushizky et al. 1960).

The second type of enzymatic reversal of ultraviolet damage is called dark repair (Setlow 1968; Howard-Flanders 1973). The enzyme system involved in this is not light activated and operates by excision of the thymine dimer and a few adjacent nucleotides. Other enzymes (DNA polymerase and polynucleotide ligase) replace the excised nucleotides restoring the strand to its state before the ultraviolet treatment.

Another type of radiation inactivation is the photodynamic action that accompanies exposure of viruses to certain dyes in the presence of visible light. Toluidine blue, methylene blue, flavins, and acridines are examples of dyes that have been employed. Since oxygen is required for, and reducing agents protect against, the effect, it appears that photodynamic inactivation is dye-mediated photooxidation (Oster and McLaren 1950; Appleyard 1967; Orlob 1967; Schaffer and Hackett 1963; McLaren and Schugar 1964). In some cases, it appears that an important effect of photodynamic action is modification of guanine residues of nucleic acids (Singer and Fraenkel-Conrat 1966).

3. *Inactivation of Viruses by Chemicals*

The main chemical agents that inactivate viruses were known many years before the detailed structures of virions and the mechanisms of virus infections were known (see review by Stanley 1938). They include enzymes, protein denaturants, oxidizing agents, acids and bases, and agents affecting primary amino groups such as formaldehyde and nitrous acid. The mechanism of inactivation by each of these agents can be readily predicted on the basis of the principle stated earlier: that infectivity may be lost by damage to the exterior of the virion of a sort that interferes with the early stages of infection (attachment, penetration, and so on) or by injury to the viral nucleic acid that prevents its complete functioning. Some treatments can cause both types of damage. Knowledge of the vulnerability of the major constituents of virions to various chemical agents is also important in predicting which mechanism is probably involved in inactivation of a given virus. Some pertinent observations of this sort follow.

a. Enzymes

Virions of different types vary in their resistance to inactivation by enzymes depending on individual structural characteristics.

Phospholipases often inactivate enveloped viruses (Franklin 1962; Simpson and Hauser 1966; Cartwright et al. 1970) by attacking the phospholipid components of the envelope and presumably disorganizing the structure required for attachment and penetration. Likwise, treatment of enveloped viruses with proteases removes glycoprotein spikes with concomitant loss of infectivity, which is probably also associated with inability of such altered viruses to attach to cells (Cartwright et al. 1970; Chen et al. 1971).

Nonenveloped viruses tend to be quite resistant to inactivation by proteases (trypsin, chymotrypsin, pepsin, papain, bromelain, pronase, and so on) unless the coat protein is denatured by some means. Poliovirus illustrates such resistance to enzymes (and to a range of pH values) by passing unscathed through the human digestive tract to establish infection in intestinal cells. However, another picornavirus similar to poliovirus, coxsackievirus type A9, does appear susceptible to partial degradation by the enzyme pronase (Herrmann and Cliver 1973). The coat proteins of some other simple viruses can be somewhat degraded by proteases and yet retain infectivity. For instance, the C-terminal threonine residues can be removed by carboxypeptidase from all of the coat protein subunits of tobacco mosaic virus without reducing infectivity of the virus (Harris and Knight 1955); and a peptide of over 2000 daltons can be digested off the coat protein subunits of potato virus X by trypsin without loss of infectivity (Tung and Knight 1972b). However, some nonenveloped viruses, such as poxviruses and reoviruses, are inactivated by proteolytic digestion. These viruses possess outer shells that are susceptible to degradation by proteases but their cores are resistant (Joklik 1966; Dales and Gomatos 1965). Even though cores (which contain the viral nucleic acid) may penetrate cells, they appear unable to carry out virus replication.

It may be noted that a kind of pseudo-inactivation can occur in which infectivity of a virus is reduced simply by formation of a complex between virus and enzyme (Stanley 1934a; Loring 1942; Arimura 1973). Upon dissociation of such complexes, infectivity is recovered. Inactivation can also be a secondary effect of proteolytic attack on the exterior of virions. For example, viruses in which removal of some coat protein exposes the viral nucleic acid to nucleases will be inactivated even when coat protein is not involved in initiating infection (for example, plant viruses).

The nucleic acids of most viruses are fairly well shielded from degradation by nucleases by coat proteins, envelopes, or both. There appear to be a few exceptions in which the coat protein subunits have a loose structure with spaces between them large enough for enzyme to penetrate. A strain of the small spheroidal cucumber mosaic virus is an example of this kind; it

loses infectivity in low concentrations of ribonuclease at pH 7.2 (Francki 1968). Obviously, if the protein coat of any virus is breached by some physical or chemical agent, the nucleic acid is liable to attack by nucleases.

b. Protein Denaturants

Some chemical reagents are deliberately used to strip the protein coat from viruses in order to isolate the nucleic acid or to determine the number and nature of polypeptides in a virion. When the purpose is to isolate the nucleic acid for further studies, precautions must be taken to avoid degradation by nucleases (deoxyribonucleases and ribonucleases for DNA and RNA, respectively). Phenol is the most commonly used reagent to dissociate virions for the isolation of nucleic acid, although the anionic detergent, sodium dodecyl sulfate (SDS), is also employed, or the two together. This has been discussed in the section on preparation of nucleic acids.

SDS is also used to dissociate the proteins of virions into their constituent polypeptide chains which then can be separated as SDS-protein complexes on acrylamide gels as described in an earlier section.

SDS is effective in solubilizing viral envelopes and hence can be used to disaggregate the more complex virions as well as the simple ones. Nonionic detergents (for example, Nonidet-P40, Tween 20, Tween 80, Sterox SL) and sodium deoxycholate, another anionic detergent, are also used to disrupt enveloped viruses (Appleyard et al. 1970; Nermut 1972; Webster and Darlington 1969; Stromberg 1972). They preserve the structure of protein constituents better than ether, chloroform, and some other organic solvents. A list of useful detergents in virology is given in Table 36.

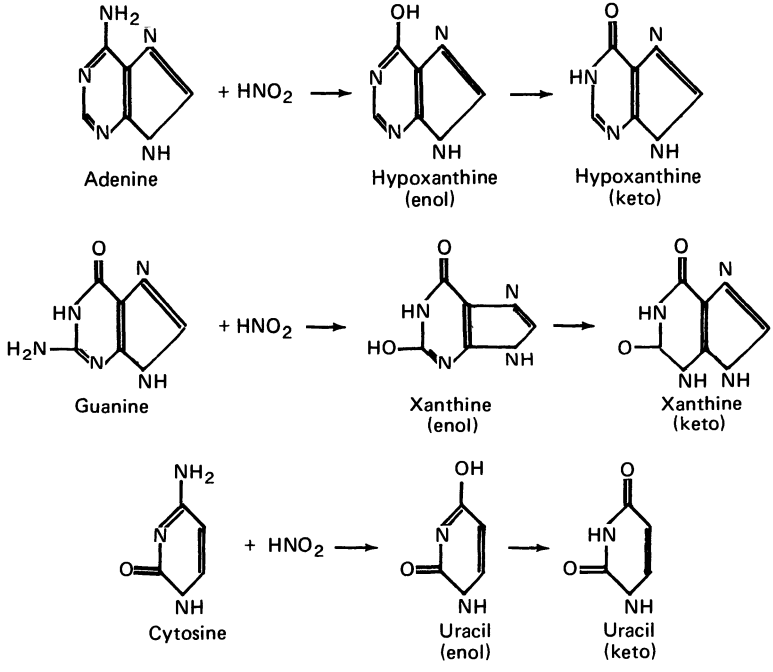
Guanidine and urea are good reagents for disrupting hydrogen bonds, which are abundant in virus particles and important in maintaining their structure. Practically all proteins are denatured by these reagents, often irreversibly.

c. Nitrous Acid

Nitrous acid can react with the primary amino groups of either protein or nucleic acid. The primary amino groups in proteins other than those at the N-terminal of the polypeptide chain, are the epsilon amino groups of lysine residues. Consequently the degree of reactivity of viral proteins with nitrous acid and the occurrence of one type of inactivation will depend considerably on the lysine content of the viral coat protein(s). In contrast, there are thousands of amino groups per molecule of any viral nucleic acid since three out of four of the constituent purines and pyrimidines have one. In the case of isolated viral nucleic acid, the accessibility of these amino groups to nitrous acid will be governed by the degree of hydrogen bonding present between complementary bases. This factor also applies to the reaction between nitrous acid and nucleic acid located inside virions to

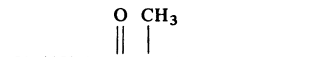
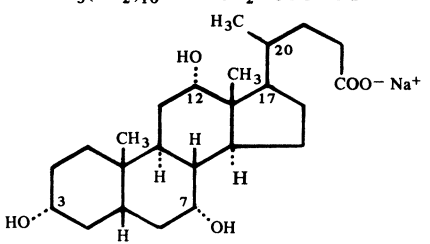
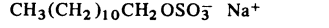
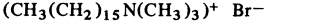
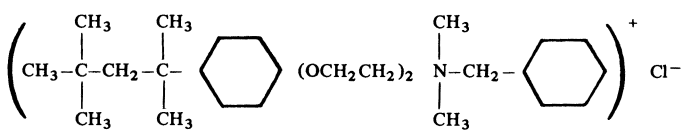
which must be added the possible complications of protein-nucleic acid interactions and the ability of nitrous acid to penetrate virions. In any case, many of the amino groups of isolated nucleic acids as well as those of nucleic acids in virions undergo reactions with nitrous acid that cause inactivation or mutation.

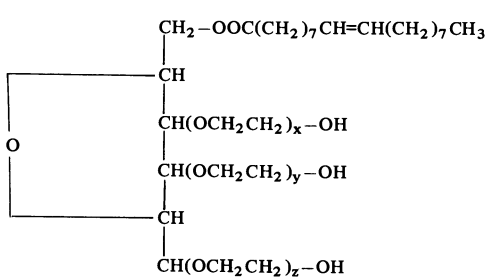
One of the the first reactions of infectious RNA to be studied was the effect of nitrous acid on infectivity (Schuster and Schramm 1958). The chemical reaction expected is the classical van Slyke (1912) oxidative deamination of primary amines: $R\text{-}NH_2 + HNO_2 \rightarrow R\text{-}OH + N_2 + H_2O$. The constituents of RNA containing primary amino groups are adenine, guanine, and cytosine (the same bases are involved in DNA with the exception that 5-hydroxymethylcytosine is substituted for cytosine in the T-even phages), and these deaminate as shown below to give hypoxanthine, xanthine, and uracil, respectively:



Schuster and Schramm (1958) showed that the deamination products indicated above were produced in TMV-RNA by treatment with nitrous acid between pH 4.1 and 4.3. These products were identified by removing samples of RNA at various times during the nitrous acid treatment, hydrolyzing, separating the products by paper chromatography, and determining the quantity of each product on the chromatograms by elution and

Table 36. Some Surfactants (i.e., Surface-active Agents or Detergents) Representative of Groups Used in Disassembling Virions.[a]

Category	Name	Chemical Formula
Anionic	1. Sarkosyl NL-97 (An N-acyl sarcosinate)	
	2. Sodium deoxycholate (A steroid)	
	3. Sodium dodecyl (lauryl) sulfate (An alkyl hydrogen sulfate)	
Cationic	1. Cetab (An alkyl quaternary ammonium salt)	
	2. Hyamine 1622 (An alkyl aryl quaternary ammonium salt)	

Nonionic	1. Alfol 16-18 (An alkylpolyethoxy alcohol)	$CH_3(CH_2)_{15\text{-}17}(OCH_2CH_2)_{12}OH$
	2. Neodol 25-12 (An alkylpolyethoxy alcohol)	$CH_3(CH_2)_{11\text{-}14}(OCH_2CH_2)_{12}OH$
	3. Sterox 67-K (An alkylpolyethoxy alcohol)	$CH_3(CH_2)_{13\text{-}14}(OCH_2CH_2)_{11}OH$
	4. Trycol TDA-12 (An alkylpolyethoxy alcohol)	$CH_3(CH_2)_{12}(OCH_2CH_2)_{12}OH$
	5. Nonidet P-40 (An alkylarylpolyethoxy alcohol)	Same as Triton X-100
	6. Triton X-100 (An alkylarylpolyethoxy alcohol)	$CH_3(CH_2)_7C_6H_4(OCH_2CH_2)_{9\text{-}10}OH$
	7. Tween 80 (Polyethoxy sorbitan monooleate)	

[a]The above agents are examples of classes of surface-active agents of which there are often many members. See Stromberg (1972) for other examples. Additional data about major classes of surface-active agents can be obtained from the manufacturers: Hyamines and Tritons, Rohm and Haas Co., Philadelphia 19105; Sarkosyls, Ciba-Geigy Corp., Ardsley, N.Y. 10502; Tweens, Atlas Chemicals Division of ICI America, Inc., Wilmington, Del. 19899; Alfols and Steroxes, Monsanto Co., St. Louis 63166. Most surfactants are not single chemical compounds but mixtures. Thus, as indicated in the table, the number of ethoxy groups or the length of a carbon chain may vary a little. The manufacturers strive for uniformity in their products so that the mixture usually contains a predominant amount of one compound and small amounts of other members of the homologous series. In the case of surfactants with fatty acid residues such as the Tweens, formulations with different properties are provided by changing the fatty acid (e.g. the fatty acid residue of Tween 20 is lauric acid, that of Tween 40 is palmitic acid, and that of Tween 80 is oleic acid). In addition, Tweens may vary in numbers of ethoxy groups incorporated and the numbers of ethoxy groups attached to different carbons in any one Tween formulation are not constant. Hence, in the formula given for Tween 80, the variable numbers of ethoxy groups are indicated by the subscripts x, y, and z. In summary, no one structural formula represents the product; instead Tween 80 is best described as a mixture of oleate partial esters of sorbitol and sorbitol anhydrides condensed with approximately 20 moles of ethylene oxide for each mole of sorbitol and its anhydrides.

spectrophotometry. In this manner it was shown that as adenine, guanine, and cytosine decreased, hypoxanthine, xanthine, and uracil increased proportionately. Significant deamination could be demonstrated after 2–4 hr at pH 4.1 and after 10–20 hours at pH 4.3 (Figure 40).

The following evidence led Schuster and Schramm (1958) to conclude that inactivation of TMV-RNA actually depends on the deamination of the purine bases and cytosine. In control experiments run at the same pH, temperature, and salt concentration but without addition of nitrous acid, the infectivity of the RNA was not lost. Hence loss of infectivity is associated with the nitrous acid treatment. Next, the possibility that nitrous acid may have caused splits in the RNA chain was investigated. No change in sedimentation coefficient could be detected after inactivation of the RNA with nitrous acid, nor could dialyzable split products be found. Hence, the most reasonable assumption is that the observed loss of infectivity is due to deamination of the bases in the RNA.

As shown in Figure 40, the inactivation of TMV-RNA with time is much more rapid at pH 4.1 than at 4.3. In both cases, the curves are of the first-order, one-hit type, indicating that deamination of a single nucleotide base is sufficient to inactivate (the aberration of the pH 4.3 curve at the beginning is due to the fact that the first two or three samples fell outside the range in which lesion numbers are proportional to RNA concentration).

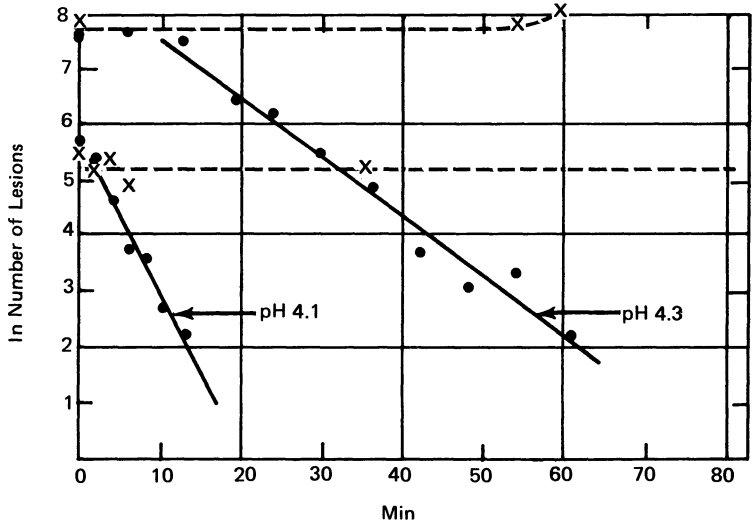


Fig. 40. Loss of infectivity of TMV-RNA with time upon treatment with nitrous acid at pH 4.1 and 4.3, respectively. Solid lines represent the nitrous acid treated samples, and dotted lines the controls held at the same pH levels and salt concentrations but not treated with nitrous acid. (Adapted from Schuster and Schramm 1958.)

According to first-order kinetics, if an average of one nucleotide base out of N nucleotides must be deaminated to produce inactivation of the RNA, then the loss of infectivity with time is given by

$$I_t = I_0 \, e^{-N\alpha t}$$

where I_t = infectivity at time t, I_0 = infectivity at time zero, and α = moles of nucleotide deaminated per minute. By changing from the exponential to the logarithmic expression and rearranging, the relation becomes

$$\ln I_0 - \ln I_t = N\alpha t$$

Then, by selecting the time when infectivity has been reduced to 37 percent (1/e), $\ln I_0 - \ln I_t = 1$ and the relation $N = (1/\alpha t)$ is obtained. N can then be found by putting in the appropriate values for t and α. Schuster and Schramm (1958) calculated α values from the results of experiments made at two different pH values in which the deamination products were determined as a function of time. In this manner, values of 3,100 and 3,500 were obtained for N. Since there are about 6,400 nucleotides in the TMV-RNA molecule, the average value of about 3,300 for N indicates that there is about a 50 percent probability that one deamination will inactivate.

Other viruses have been inactivated with nitrous acid, among them poliovirus (Boeyé 1959). An interesting contrast was noted between polio and TMV, both of which are RNA-containing viruses, in that TMV-RNA is more rapidly inactivated by nitrous acid than whole virus, whereas with poliovirus the reverse was observed. Evidence that the polio protein is involved in the inactivation process (presumably by chemical changes in the protein that reduce significantly the ability of the viral RNA to be released) can be inferred from the decreasing slope of the whole virus inactivation curve. A similar result was observed with the spheroidal southern bean mosaic virus (Sehgal 1973) where it was found that the intact virus was inactivated twice as fast as the isolated RNA even though nucleic acid bases were deaminated to the same extent.

The cases of poliovirus and southern bean mosaic virus just cited suggest involvement of the protein in the inactivation, but a peculiar instance has been reported for tobacco rattle virus. Like the other two examples, the rate of inactivation of whole virus was substantially greater than that of isolated nucleic acid but there was no indication that the viral protein was involved (Robinson 1973). There appears simply to be an enhanced inactivation of RNA *in situ* by an unknown mechanism.

Deamination studies with phages have helped to evaluate the relative significance of the amino-purine and amino-pyrimidine bases in inactivation and mutation induced by nitrous acid. Vielmetter and Schuster (1960) treated T2 phage with nitrous acid for up to 60 hr and isolated and analyzed the DNA from aliquots at various times. As shown in Table 37, there is a great difference in the rate of deamination, depending upon pH, but at both

Table 37. Rates of Deamination of Bases in Coliphage T2 DNA by Treatment with Nitrous Acid at pH 4.2 and 4.5 Together with Rates of Inactivation and Production of Plaque Type Mutants.[a]

	Deaminations per Minute ($\times 10^{-6}$)	
	pH 4.2	pH 5.0
Adenine	18.5	<0.2
Guanine	297	8.6
5-Hydroxymethylcytosine	117	1.3
Lethal hits per minute	9	0.3
$\frac{\%\ r^-\ \text{mutants per minute}}{\text{survivors}}$	2.9	3.3×10^{-2}

[a] Adapted from Vielmetter and Schuster 1960.

pH 4.2 and 5.0, guanine in T2-DNA was deaminated much more rapidly and adenine much less quickly than 5-hydroxymethylcytosine. Schuster (1960b) observed this same relationship for the deamination of the bases for DNA from a variety of sources. However, which bases are involved in deamination can apparently be dependent on conformation and structural interactions of the nucleic acid with its environment. This was illustrated by the results of studies on TMV in which it was shown that treatment of isolated viral RNA with nitrous acid results in deamination of adenine, guanine, and cytosine but that a similar treatment of whole virus deaminates two of the three bases, guanine escaping attack (Schuster and Wilhelm 1963). A corollary is the finding that at the same level of survival, more mutants are obtained by treating whole virus with nitrous acid than by the reaction with free nucleic acid (Sehgal and Krause 1968).

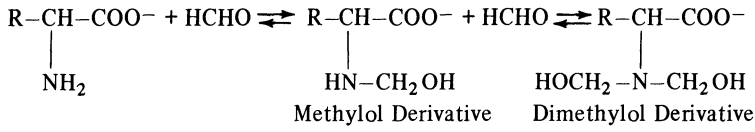
The hydrogen bonding between complementary strands of DNA involves amino groups of purines and pyrimidines (see Figure 24) and greatly reduces the capacity of such groups to react with nitrous acid, formaldehyde, and other reagents. However, at the pH values used in the reaction with nitrous acid, it appears that incipient separation of strands occurs, permitting a limited reaction with small molecules (Singer and Fraenkel-Conrat 1969). It has also been suggested that the numerous folds required to pack viral DNA into a phage head (T4 DNA is about 600 times longer than the T4 phage head) may cause distortions of structure that expose some of the reactive groups of the purine and pyrimidine bases (Freese and Strack 1962).

Apparently, inactivation can also be caused by nitrous acid and other reagents through formation of covalent cross-links between complementary strands of nucleic acid or even between loops of single-stranded nucleic acid (Becker et al. 1964).

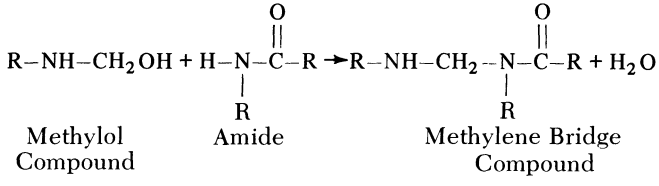
Whatever the specific chemical change in viral nucleic acid produced by treatment with nitrous acid, if inactivation occurs it can be assumed that one or more of the vital functions of the nucleic acid is being blocked: (1) template for the replication of the nucleic acid, (2) transcription, or (3) translation. This same principle applies of course to changes in the nucleic acid brought about by a wide variety of agents.

d. Formaldehyde and Other Aldehydes

The inactivation of viruses by formaldehyde has been known for years, and is of particular importance because of its wide application in the production of vaccines containing noninfectious but antigenically active viruses (Potash 1968). It was long supposed that formalin inactivation primarily involved reaction of amino groups of the viral protein with formaldehyde to yield mono- or dimethylol derivatives, which can be illustrated as follows with an amino acid:



This reaction is the basis of the classical Henriques-Sorenson (1910) formol titration method. However, the reaction of proteins with formaldehyde is more complex than the above, and it was shown, for example (Fraenkel-Conrat and Olcott 1948), that at room temperature and over a wide pH range cross-linking methylene bridges can be formed between aminomethylol groups, on one hand, and amide or guanidyl groups, on the other. Thus, after the initial fast reaction of a primary amino group with formaldehyde to form a methylol compound, a slower cross-linking reaction may occur as follows:



The formation of methylol compounds is, as indicated, a reversible reaction, whereas the cross-linking reaction is much less so and is now regarded as contributing greatly to the tanning or hardening action of formaldehyde on proteins.

These reactions doubtless occur to a variable extent with viruses treated with formaldehyde, but a new interpretation of the formaldehyde reaction

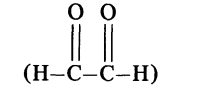
is suggested by the knowledge that nucleic acid is the seat of viral infectivity and that nucleic acid reacts with formaldehyde (Fraenkel-Conrat 1954). It now seems that inactivation of viruses by formaldehyde involves both protein and nucleic acid. The primary inactivating effect depends on the reaction of formaldehyde with the nucleic acid, but sufficient alteration of the protein coat by the cross-linking reaction described above may prevent release of nucleic acid in the infected cell, and hence may also contribute to the inactivation. In this connection it is conceivable that the protein may be significantly altered before the nucleic acid is rendered noninfectious.

Quantitative studies on the reaction of TMV-RNA with formaldehyde and other aldehydes have been made by Staehelin (1958, 1959, 1960), using ^{14}C-labeled compounds. It was expected that formaldehyde would react only with bases containing amino groups (adenine, guanine, and cytosine) and this seems to be the case. At least the reaction with formaldehyde stops at about the level of 60–70 moles of formaldehyde per 100 moles nucleotide, which is about the proportion of adenine, guanine, and cytosine (the amino bases) to total nucleotides present. The extent of reaction was found to be diminished by changing the phosphate buffer concentration from 0.001 M to 0.1 M or by adding divalent cations, presumably because these conditions favor formation of secondary structure and thus reduce the availability of the amino groups.

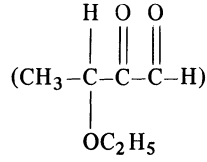
In contrast to isolated RNA, intact TMV was found to be inactivated only when about 100 times greater concentrations of formaldehyde and longer times of treatment were employed. This suggests that the amino groups of the RNA are hydrogen bonded with protein in the intact virus and therefore are less accessible, or that the formaldehyde has some difficulty in penetrating the protein coat to reach the deeply embedded RNA. Since the inactivation of TMV by aldehydes was observed to proceed less readily with larger aldehydes than with formaldehyde, whereas this was not so in the inactivation of free RNA, Staehelin (1960) suggests that penetration difficulties probably explain the difference in reactivity between RNA and intact virus.

A detailed review of the reaction of formaldehyde with nucleic acids and their constituents was written by Feldman (1973).

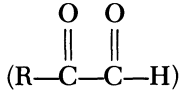
The reaction of TMV-RNA with glyoxal derivatives was found to differ strikingly from its reaction with formaldehyde as judged by effect on ultraviolet absorption (Staehelin 1959). In contrast to the shift of the absorption maximum to a higher wavelength (3–5 nm) and an increase in absorption with time (as much as 30 percent) with formaldehyde, treatment with glyoxal



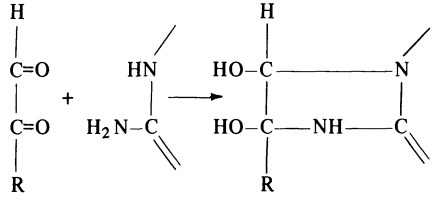
or kethoxal



caused a very small increase in absorption and only a very slight shift in wavelength of the maximum absorption, and this toward the smaller wavelengths. By using radioactive kethoxal, it was shown that guanylic acid is the only one of the four RNA nucleotides that reacts with glyoxal derivatives:



It was suggested that the reaction of nucleic acid or its guanine derivatives with compounds related to glyoxal might involve formation of stable five-membered ring structures as shown here:



Reaction between TMV-RNA and stable glyoxal derivatives such as kethoxal (glyoxal itself is unstable) takes place more readily and much faster than with formaldehyde, as shown in Table 38.

Kethoxal has been applied to several animal viruses with the following results (Renis 1970: (1) three picornaviruses–polio, coxsackie, and encephalomyocarditis viruses–were not activated by kethoxal although the isolated RNA of coxsackie virus was; (2) reovirus-3 and pseudorabies virus were moderately inactivated; (3) herpes, influenza, parainfluenza-3, Newcastle disease, Sindbis, and vesicular stomatitis viruses were strongly inactivated.

e. Hydroxylamine

The inactivation of bacterial, animal, and plant viruses by hydroxylamine has been reported (Kozloff et al. 1957; Freese et al. 1961a; Franklin and Wecker 1959; Holland et al. 1960; Schuster 1961). Some of the outstanding features of the reaction of viral nucleic acids with hydroxylamine are as follows:

Table 38. Inactivation of TMV-RNA by Aldehydes.[a,b]

Aldehyde	Concentration %	Activity %
Formaldehyde	0.1	0
	0.05	4
	0.025	34
Glyoxal	0.1	6
	0.05	23
	0.025	81
	0.0125	100
Kethoxal	0.02	1
	0.01	8
	0.005	32
	0.0025	73

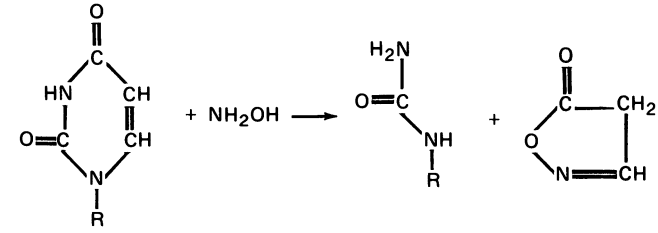
[a]From Staehelin 1959.

[b]TMV-RNA at 1 mg/ml was treated with the aldehydes indicated for 30 minutes at about 23° in 0.001 M phosphate buffer at pH 6.8. After the reaction, the nucleic acid was precipitated 6 times with alcohol to remove unreacted aldehyde and then assayed.

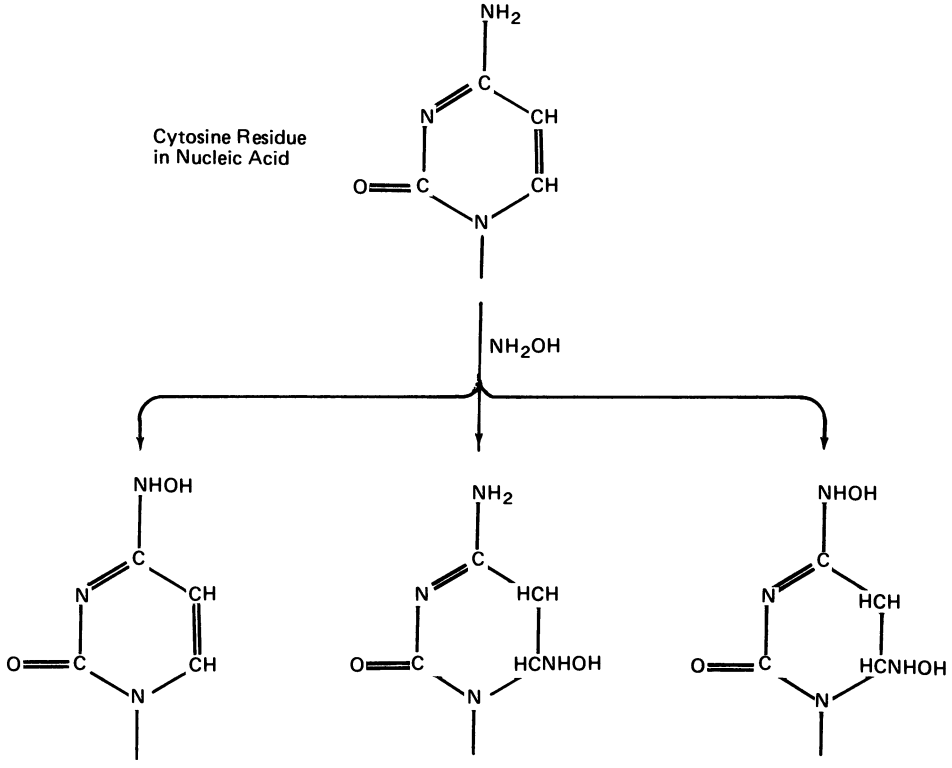
1. No decrease in molecular weight of the nucleic acid occurs under conditions that lead to complete loss of infectivity. Hence, a reaction with the nucleic acid bases is indicated.
2. Hydroxylamine seems to react only with pyrimidines and not with purines (Schuster 1961; Freese et al. 1961b).
3. Among the pyrimidines the reaction rate varies with pH and the nature of substituents present in the pyrimidine ring. The reaction can be detected by change in ultraviolet absorption and also chemically, in due time, by loss of pyrimidine and gain in certain reaction products (see below).

In RNA, cytosine was found to react with hydroxylamine at pH 6 at least 30 times faster than uracil, whereas at pH 9, uracil reacts at least 8 times faster than cytosine (Schuster 1961). The reaction proceeds best (Schuster 1961) if carbon atoms 5 and 6 (or C atoms 4 and 5 by Fischer system) are unsubstituted, and pyrimidines with substituents on these atoms react only slightly or require more vigorous treatment (higher concentration of hydroxylamine and higher temperature). This point is illustrated by the data in Table 39 which show the effect on ultraviolet absorption of treatment with hydroxylamine at a pH of 7.5, a value intermediate to the optima for cytosine and uracil.

The reaction of hydroxylamine with uracil appears to destroy the pyrimidine ring yielding an isoxazolone fragment and a ribosyl urea residue. The primary reaction proposed by Schuster (1961) is as follows:



The reactions of hydroxylamine with cytosine residues in nucleic acids (Phillips and Brown 1967) may be represented as follows:



It is not yet clear which products of the action of hydroxylamine are inactivating and which are mutagenic, although the reaction with uracil and those with cytosine in which hydroxylamine is added to the 5-6 double bonds to produce the two structures shown on the right above are considered the most likely inactivating events (Singer and Fraenkel-Conrat 1969). In addition it has been proposed that inactivation by hydroxylamine under

Table 39. Decrease of Ultraviolet Absorption of TMV-RNA and of Pyrimidines and Derivatives upon Treatment with Hydroxylamine.[a]

Compound	Wavelength, nm	Percent Decrease in Absorption
Adenylic acid	260	0
Guanylic acid	250	0
6-Aminocytosine	260	1
Thymidylic acid	270	1
TMV-RNA	260	2
5-Methylcytosine	270	3
Orotic acid	280	4
Cytidine	270	12
Uracil	260	19
Cytosine	265	20
Uridine	260	27
Thymidine	265	15[b]

[a]Adapted from Schuster 1961.

[b]Thymidine was treated with 2 M NH_2OH at pH 7.5 for 15 hr at 45°; all other reactions were with 1 M NH_2OH at pH 7.5 for 15 hr at 20°.

aerobic conditions may involve the action of free radicals (Freese and Freese 1965). The reaction of hydroxylamine with the amino group on C4 of cytosine to produce the product shown on the left above (and its tautomeric form) is considered a likely mutagenic event.

f. Alkylating Agents

Alkylating agents are chemicals that can introduce alkyl groups into proteins and nucleic acids, the current emphasis being on the latter. They may be monofunctional, di-, or polyfunctional depending on the number of reactive groups they contain. A review (Lawley 1966) and a book (Loveless 1966) provide many details about the chemistry and genetic effects of alkylating agents.

The first chemical mutagen to be reported was mustard gas, an alkylating agent (Auerbach and Robson 1947). Ethyl methane sulfonate was apparently the first alkylating agent applied to a virus, which happened to be a bacteriophage (Loveless 1958); and a rather extensive study of the effect of alkylating agents on TMV was made a few years later (Fraenkel-Conrat 1960).

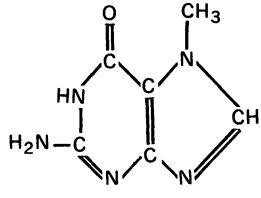
Several alkylating agents were used on TMV and TMV-RNA (Fraenkel-Conrat 1960), and the use of such agents tagged with radioisotopes enabled the sites of reaction to be determined in several cases. As indicated in Table 40, all of the reagents seemed to react principally with guanine, although iodoacetate alkylated adenine as well. Where it could be

Table 40. Effect of Some Alkylating Agents on TMV-RNA.[a]

Reagent	Group Introduced	Site of Reaction	Inactivation of RNA (groups per mole RNA to reduce infectivity to 37%)	Mutational Effect
Iodoacetate	$-CH_2-\overset{\displaystyle O}{\overset{\|}{C}}-O^-$ (carboxymethyl)	A, G	1–3	–
Ethylene oxide	$-CH_2-CH_2-OH$ (hydroxyethyl)	G	1–3	–
Propylene oxide	$CH_3-\underset{\displaystyle \|}{CH}-CH_2OH$ (hydroxyisopropyl)	?	?	–
Mustard gas (bis-β-chloroethyl sulfide)	$-CH_2-CH_2-S-CH_2-CH_2-OH$ (β-hydroxyethyl-thioethyl)	G	?	–
Dimethyl sulfate	$-CH_3$ (methyl)	G	?	+

[a]Data from Fraenkel-Conrat 1960.

measured, it appeared that introduction of one to three alkyl residues into the RNA caused a reduction in infectivity to the 37 percent level. Only one of the reagents, dimethyl sulfate, appeared to be consistently mutagenic. From RNA allowed to react with dimethyl sulfate much longer than required for inactivation, it was possible to isolate 7-methyl guanine:



Studies of alkylations of other viruses and nucleic acids have confirmed and extended these observations (see Drake 1970; Freese 1971; Singer and Fraenkel-Conrat 1969). The following general conclusions can be stated. Alkylating agents attack principally the ring nitrogens of the purine and pyrimidine bases, predominantly N7 of guanine, and to variable smaller extents, depending on type of reagent and of nucleic acid, the N1, N3, and N7 positions of adenine and N3 of pyrimidines.

Inactivation is expected to occur when alkylation affects base pairing atoms such as N1 of adenine and N3 of pyrimidines. When the alkylating agent is polyfunctional like the sulfur or nitrogen mustards, there is a possibility of cross-linking between complementary strands or segments of nucleic acids. Such cross-linking is probably inactivating. Likewise, depurination, which is a possible side reaction, is inactivating. Mutational possibilities following alkylation will be considered later.

g. Other Inactivating Chemicals

Strong acids and bases inactivate viruses because they are able to damage both protein and nucleic acid. Proteins vary considerably in their resistance to acid denaturation, those of poliovirus being quite resistant (Loring and Schwerdt 1944), whereas those of myxoviruses and rhinoviruses are very susceptible (Miller 1944; Dimmock and Tyrrell 1962). Most viruses are disrupted by alkali with or without denaturation of protein constituents (Stanley 1938). RNA is degraded by alkali while DNA is resistant; both types of nucleic acid are attacked by acids, loss of purine bases (depurination) often resulting and degradation of the polynucleotide chains occurring to an extent determined by conditions (Loring 1955).

It has been recognized for years that oxidizing agents inactivate viruses (Stanley 1938). Examples of oxidizing agents tested on viruses include peroxide, iodine, other halogens (especially hypohalites), and, in the case of the most sensitive viruses, just air. A major structural effect of hydrogen

peroxide on T-even phages is the inducing of these phages to eject their DNA (Kellenberger and Arber 1955). The oxidative deamination by nitrous acid was considered earlier.

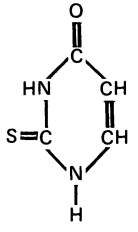
Halogenation is generally inactivating with nucleic acid as a prime target (Brammer 1963). In experiments with TMV-RNA, adenine and uracil were resistant to halogenation but *N*-bromosuccinimide reacted with both guanine and cytosine at pH 7 and pH 9, although reaction with guanine was more favored at pH 9. Bromine gave a more specific reaction, reacting almost exclusively with cytosine at pH 7 and with guanine at pH 9. Mutagenic action has been reported for both *N*-bromosuccinimide and bromine (Singer and Fraenkel-Conrat 1969).

It can be reiterated here that any chemical that irreversibly denatures protein or that alters nucleic acid in such a way as to interfere with its use as a template will either inactivate or mutate.

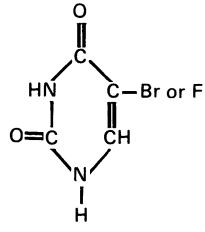
h. *In Vivo* Inactivators of Viruses

The inactivating agents discussed above were considered primarily on the basis of their application to viruses in the laboratory and the results of subsequent tests in appropriate hosts. Much has been learned from such studies, but there is a growing concern about what kinds of inactivating and mutating influences occur or can be induced in cells. A few kinds of intracellular inactivations of viruses will be considered here.

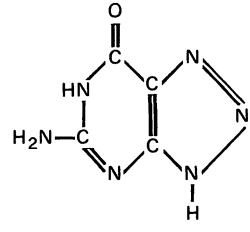
1. Base analogs. It was natural when the primacy of nucleic acid in the reproduction of viruses became apparent that metabolic antagonists should be tested for effect on virus growth. Consequently, numerous purine and pyrimidine analogs have been added to viral systems and the effects noted (see Matthews and Smith 1955). Among the analogs studied are the following:



2-Thiouracil



5-Bromouracil
and 5-Fluorouracil



8-Azaguanine

Some analogs show strong inhibitory effects on virus multiplication and others not. Some of the compounds effective with one virus are ineffective with another. Certain viruses such as T2 phage, not directly inactivated by incorporation of a pyrimidine analog, may be substantially more sensitive

than normal phage to subsequent exposure to x-rays, ultraviolet light, or even visible light (Stahl et al. 1961). Among explanations offered for this effect are the greater absorption of radiation by bromouracil as opposed to thymine and the possibility that radiation damage to the analog may not be as readily reversed by repair enzymes as damage to thymine.

Some analogs have been shown to be incorporated into viral nucleic acids by subsequently isolating the analog-containing nucleotides. For example, although 5-fluorouracil (or better yet, 5-fluorouridine) inhibits the multiplication of TMV, the virus does multiply in the presence of this analog and replaces as much as 30–50 percent of the uracil with the 5-fluorocompound (Gordon and Staehelin 1959). This is equivalent to about 500–800 molecules of 5-fluorouracil per mole of RNA. Nevertheless, such altered virus was found to be fully infective on Xanthi nc tobacco. On the other hand, 2-thiouracil and 8-azaguanine, both of which are incorporated into viral RNA, inhibit the multiplication of TMV and other plant viruses (Matthews and Smith 1955; Loebenstein 1972).

The base analogs shown to inhibit multiplication of animal viruses are primarily the 5-halogenated uracils, and inhibition seems most effective when these are applied as the uridine derivatives; however, other compounds such as 2-thiouridine and 2-aminopurine have also been used in animal virus systems (Prusoff et al. 1965; Kaplan and Ben-Porat 1970; Sambrook et al. 1966; Slechta and Hunter 1970).

Animal viruses whose multiplication has been blocked by base analogs include rabbitpox, pseudorabies, polyoma, herpes simplex, and coxsackie viruses. Two mechanisms of action are considered most likely to explain the antiviral effects observed: (1) incorporation of analog causes lethal mistakes in some protein coded by the viral nucleic acid (that is, an amino acid is replaced by one that renders the protein nonfunctional); (2) the analogs react with and inactivate some enzyme needed for virus replication.

Base analogs are also mutagenic but this will be considered in the next section.

2. *Radiophosphorus.* It is possible to incorporate a self-destruction mechanism (so-called "^{32}P suicide") into viral nucleic acid by supplying virus-infected cells with ^{32}P phosphate of high specific activity. Such radioactive phosphorus is incorporated into nascent nucleic acid as the virus replicates. Since all nucleic acids are characterized by a linear structure in which hundreds of nucleotides are linked by phosphodiester bonds (see small segment of nucleic acid illustrated in Figure 17), decay of a ^{32}P atom to ^{32}S (with release of a beta particle) may result in rupture of the nucleic acid strand at the site of decay. The break apparently reflects the inability of the sulfodiester linkage that replaces the phosphodiester bonding to withstand the energy exchanges accompanying the transmutation.

Inactivation by ^{32}P decay is a one-hit phenomenon—whether observed

with double-stranded DNA of T2 coliphage (Hershey et al. 1951; Stent and Fuerst 1955, 1960), polyoma virus (Benjamin 1965), with single-stranded DNA of coliphage ØX174 (Sinsheimer 1959b), or single-stranded RNA of poliovirus (Henry and Youngner 1963). The fraction of survivors (S) is proportional to the number of incorporated ^{32}P atoms that have decayed by the time of assay for infectivity according to the formula $S = e^{-\alpha N f}$, where α is the efficiency of inactivation per ^{32}P disintegration, N the average number of ^{32}P atoms per nucleic acid molecule, and f the fraction of ^{32}P atoms decayed by a given time. A plot illustrating the survival of ^{32}P-labeled phage T2 with time is given in Figure 41. From information available, α, the efficiency of inactivation, can be calculated. Such calcula-

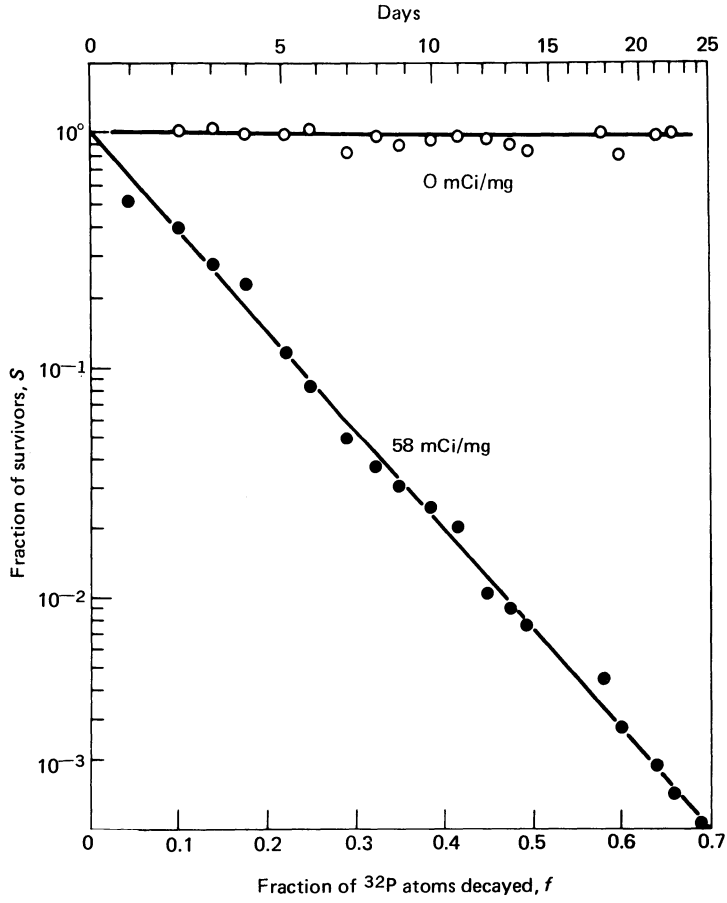


Fig. 41. Survival of ^{32}P-labeled and nonlabeled stocks of T2 bacteriophage stored at 4°C, as a function of the fraction of the ^{32}P atoms that have decayed by the day of assay. (Adapted from Stent and Fuerst 1960.)

tions indicate that only about one in ten disintegrations of P result in inactivation of T2 phage, whereas ØX174 DNA or polivirus RNA are inactivated with an efficiency near 1. This difference seems related to the necessity to have disintegrations in the same vicinity on complementary strands of double-stranded nucleic acid in order to break the molecule, whereas a break anywhere does it for single-stranded nucleic acid.

The situation is actually more complex than this (Harriman and Stent 1964); for example, a break in one strand of a double-stranded molecule may be readily repaired thus restoring full function since the molecule remains together with a nick in it. Conversely, a break in only one strand may be lethal if by chance it is in a gene coding for a needed repair enzyme. Nevertheless, there is a distinctive difference in the efficiency of inactivation of single-stranded and double-stranded nucleic acids by ^{32}P decay.

3. *Antibodies and interferons.* Vertebrates have at least two mechanisms for inactivating viruses not present in the cells of protists or plants. These are antibodies and interferons.

Antibodies are specific proteins (immunoglobulins) produced by special cells (lymphocytes) in response to foreign substances introduced into the animal. In general, viruses are good antigens (antigens are substances that elicit the formation of antibodies and react specifically with them). Consequently, recovery from a virus disease often leaves an animal immune for some time to further attack from the same or closely related strains of virus, and such immunity can usually be correlated with the presence of specific immunoglobulins (antibodies) in blood, lymph, and tissues.

This situation is exploited in the production and use of prophylactic vaccines. In some cases a "live" vaccine is employed that consists of a mild strain of virus that gives a localized infection; antibodies are induced to the mild strain that also neutralize more virulent strains. Carefully inactivated ("killed") virus is injected in a second type of vaccination. Virions that are not infectious but still antigenic can be obtained by proper treatment of virus with such agents as formaldehyde, ethylene oxide, β-propiolactone, or ultraviolet light. In some cases, for example, in some influenza vaccines (Ruben and Jackson 1972), the virions are dissociated by a surface active agent to release antigenic components of the virus, which are separated from other materials and concentrated by column chromatography for use in the vaccine. Detailed descriptions of various methods for vaccine preparation are given by Potash (1968). An excellent exposition on immunoglobulins by many specialists in various aspects of the topic is available (Kochwa and Kunkel 1971).

Viruses can be inactivated by their reaction with antibodies according to two mechanisms: (1) With some viruses the virus-antibody complex cannot attach and penetrate cells and thus such virus fails to get an infection started; (2) the complex is taken up by macrophages (scavenger cells

present in various tissues) by a phagocytotic process, or in some cases, in contrast to above, the complex is engulfed by cells normally susceptible to free virus. Within macrophages or in phagocytic vesicles of other cells, the virus is degraded enzymatically. Virus-antibody complex appears more readily degraded than free virus, which can also be engulfed by macrophages.

It should be noted that inactivating ("neutralizing") antibody does not irreversibly inactivate virus simply by forming a complex with it; virus-antibody complexes can be dissociated by dilution, treatment with salts, dilute alkali, and so on with release of infectious virus.

The immune reactions of viruses with tissues and cells represent a complex area with gaps in understanding, but many aspects are dealt with in a review by Mims (1964).

Interferons are substances whose name is derived from the observation first made by Isaacs and Lindenmann (1957) that treatment of chick cells with heat-inactivated influenza virus causes release of a soluble factor that interferes with the infection of other cells. Interferon seems to be a glycoprotein of about 30,000 daltons that is heat resistant and stable over a wide pH range (Colby 1971; Kleinschmidt 1972).

Interferon is species specific but not virus specific. This means that interferon produced in a given type of cell such as in chick cells is active against a wide variety of viruses presented to chick cells but has little effect against the same viruses in mouse or monkey cells. This and other facts suggest the nature of interferon, namely, that it is a gene product of the host rather than of the virus. Hence there are many interferons each characteristic of the species in which it is produced.

Interferon does not inactivate viruses when mixed with them nor does it interfere with the attachment, penetration, or uncoating of infecting viruses. Interferon appears to be an inducer of another host-coded protein, "virus inhibitory" or "antiviral" protein. This antiviral protein is thought to interfere with translation of viral mRNA but not with host mRNA; however, there is also evidence that it may react with virus-specific RNA polymerase to prevent its function.

It might seem that interferons and/or antiviral proteins would be ideal antiviral agents either administered directly or induced in the desired host. However, at present it is impractical to get enough homologous interferon, or less yet, of antiviral protein to inject into people or other animals; similarly it is not yet feasible to induce interferon or antiviral protein in the cells where it is required. A good discussion of techniques for the study of interferons and related matters has been published (Wagner et al. 1968).

4. Miscellaneous antiviral substances. Prodigious efforts have been made to find antiviral (chemoprophylactic) drugs that might equal for viruses the success of antibiotics against bacteria. Limited success has been achieved in this objective, although the rationale for production of antiviral

drugs is clear: disrupt the synthesis or function of viral nucleic acid or protein(s) without serious disturbance of host cell metabolism.

Many chemicals, belonging however to a restricted number of types of compounds, have been found to inhibit the multiplication of different animal viruses in cell cultures [see Whipple (1965) and Herrmann and Stinebring (1970) for an extensive review of antiviral substances]. These include nucleoside derivatives of many kinds, thiosemicarbazones, amantadine derivatives, thiazolidines, guanidine, and isoquinolines. However, when it comes to clinical trials with the whole animal, many chemicals prove to be ineffective virus inhibitors, produce unpleasant or even toxic side effects, or induce both resistant and dependent mutants. Only two types of antiviral compounds have shown impressive efficacy without significant toxicity or side effects. They are halogenated deoxyribosides (but see below) and amantadine derivatives (see their basic structures illustrated in Figure 42).

Halogenated nucleosides, especially deoxyribosides such as 5-iodouracildeoxyriboside (IUDR or idoxuridine), are effective inhibitors of several viruses containing DNA. A halogenated nucleoside such as IUDR is converted in the cell to the nucleoside triphosphate and incorporated into DNA in place of thymine as the viral DNA replicates. This may cause mispairing in further replication and faulty transcription of the DNA strand containing halogenated pyrimidine. Thus the replication and function of

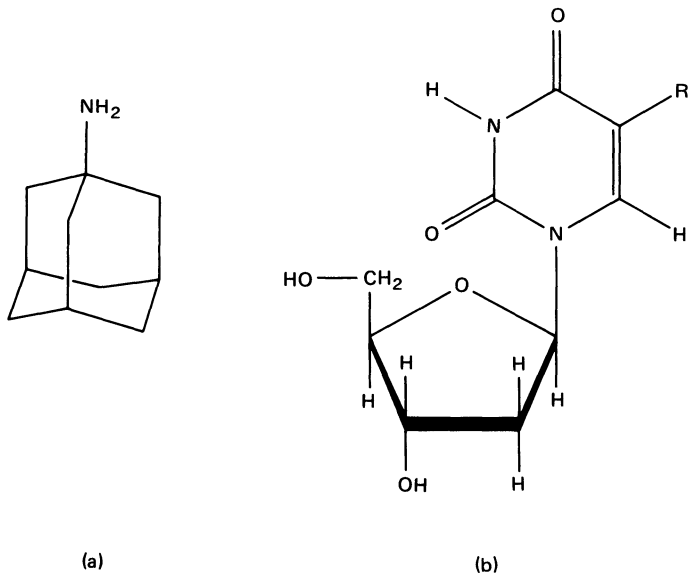


Fig. 42. Basic structural formulas for two types of antiviral drug. *a*. Amantadine (1-adamantanamine); *b*. halogen-substituted deoxyuridine where R is Br, F, or I.

viral DNA are both impaired. These are not specific effects on the virus but affect cellular DNA replication and function as well. For example, after intravenous infusion of IUDR for 6 days at a total dosage of 520 mg/kg of body weight, an adult patient showed a reduction in leucocytes and platelets, abnormal liver function, and bloody diarrhea (Tomlinson and Mac Callum 1970). Obviously, halogenated nucleosides can have marked toxic effects. However, IUDR is effective and nontoxic in topical applications to the eye infected with herpes simplex or vaccinia viruses (Kaufman 1965).

Undoubtedly, the most successful antiviral agent in clinical use at present is amantadine and its derivatives [the synthesis and testing of 37 derivatives have been described by May and Peteri (1973)]. They are used as chemoprophylactic agents against influenza A and certain strains of parainfluenza (see Galbraith et al. 1970). Amantadine and its derivatives are not viricidal but appear to interfere with viral penetration into host cells and possibly uncoating after entry.

B. Mutation

A virus mutation may be defined as a heritable change in the nucleic acid (also called the genome or genotype) of the virus. The product of mutation is called a mutant, or variant, or, especially with plant viruses, a strain.

Mutants of all types of viruses are recognized by distinctive heritable features, that is, by changes in the phenotype. There are many such characteristics, but most commonly mutants are recognized by differences in (1) host or tissue specificity, (2) disease symptoms, (3) serological specificity, and (4) protein structure. In some cases it is possible to demonstrate directly mutational changes in the genotype, that is, in the structure of the nucleic acid itself. It should be noted that the consequences of mutation are not always readily detected, and that some mutants are viable and hence detectable only under a special set of conditions.

For example, a mutant may multiply in one host (permissive host) but not in another (nonpermissive or restrictive host), whereas the parental virus multiplies in both. Likewise a temperature-sensitive (ts) mutant may replicate at a certain temperature and not at a higher temperature, while the parental virus multiplies at both temperatures. Since the host-restricted and ts mutants cannot multiply under conditions in which the common or wild type virus can, they are often referred to as conditional lethal mutants; a lethal mutant is the product of mutation not able to replicate under any known conditions. While mutation is essentially a discontinuous process, occurring in discrete steps, repeated mutations can occur. Consequently, mutants are often observed that display accumulated changes in characteristics.

It is probably safe to say that all viruses are capable of mutation and do mutate spontaneously in nature. The frequency of mutation (mutation rate) is difficult to ascertain with certainty owing to the difficulty of detecting and scoring accurately the great number of variations that can occur. However, fairly accurate estimates can be made of the frequency with which specific types of mutants arise (see chap. 5 in Drake 1970; Stent 1971). For example spontaneous mutation of T2 coliphage may produce plaque type (r) mutants at a frequency of about 1 in 10^4 duplications of the phage, whereas mutants of the host-range type (h) may appear with a frequency of only 1 in 10^8 duplications of the phage. These results can be expressed respectively as probabilities of 10^{-4} and 10^{-8} mutations per phage per duplication (Stent 1971).

A similar range in frequency of spontaneous mutations has been observed with several RNA-containing animal viruses (Dulbecco and Vogt 1958; Takemori and Nomura 1960; Carp 1963; Breeze and Subak-Sharpe 1967; Medill-Brown and Briody 1955). Reliable calculations of spontaneous mutation rates seem not to have been made for DNA-containing animal viruses. However, some DNA animal viruses such as rabbitpox virus yield pock type mutants with a frequency as high as 1 percent (Gemmell and Fenner 1960). One interpretation for this high frequency is that individual mutations may occur at a frequency of only 10^{-5} but at a thousand separate genetic sites.

Only crude estimates of spontaneous mutation rates have been made for plant viruses. However, some cases seem to be high like the rabbitpox virus cited above. For example, Kunkel (1940) found about 0.5 percent mutants in the tobacco mosaic virus present in sap of plants that had been inoculated five to six weeks earlier with a pure culture of common or wild type virus.

The mutation rates just discussed are for forward mutation. When a mutant replicates it can experience other forward mutations, or mutations can occur that restore the previous phenotype. The latter events are called back mutations, or reversions. It is a true reversion if back mutation occurs at the original site, but if the lost or altered characteristic is restored by mutation at a site separable from the original one by recombination, it is called a suppressor mutation. The frequency of back mutations has not been determined accurately in many cases but those observed with coliphage T4 (Drake and McGuire 1967) vary over a tremendous range (at least 10^{-4}–10^{-11}). In both forward and back mutations the observed frequencies must reflect the molecular mechanisms involved in the mutational event. Some of these mechanisms will be considered shortly.

Various procedures are used experimentally for isolating mutants from a population of viruses. Occasionally, selection of a mutant can be accomplished by passage in a host that is different from the one favoring the wild type. More commonly, use of techniques that yield isolated colonies

(clones) of virus are employed. Thus pure cultures of mutants as well as of wild type are obtained from plaques formed by phages on lawns of bacteria, from plaques of tissue culture cells infected with animal viruses, or from local lesions or other distinctive spots on leaves of virus-infected plants. Such virus colonies are illustrated in Figure 3.

1. Molecular Mechanisms of Mutation

Viral nucleic acids exhibit considerable plasticity in that they can withstand many changes without loss of function, and can undergo some alterations that permit function under special conditions (conditional lethal mutants). Many changes are, of course, inactivating, that is, lethal.

Two types of alterations of viral nucleic acids appear to be the major causes of mutations: (1) nucleotide substitutions (also called base substitutions since a mutational change in a nucleotide is usually accomplished by a change in the purine or pyrimidine base of the nucleotide); (2) addition or deletion of one or more nucleotides. In addition, there is some evidence for rearrangement of large polynucleotide segments through inversion (see Drake 1970); and, although not commonly considered as such, the genetic hybrids resulting from recombination between the nucleic acids of two viruses, or between the nucleic acids of a temperate virus and its host, satisfy major criteria for mutants.

The main events of the central dogma scheme (replication, transcription, and translation of nucleic acid) all depend on the phenomenon of base pairing illustrated in Figure 24. Hydrogen bonding of H with contiguous N or O atoms is the key to specific base pairing. Thus adenine pairs with thymine in the replication process and with uracil in transcription, while guanine pairs with cytosine; and this specific pairing generally occurs with great fidelity. However, the structures of nucleic acid bases permit migration of hydrogen atoms from one atom to an adjacent one (tautomeric shift) so that different pairing arrangements can plausibly occur. For example, Figure 43 illustrates how the shift of a proton from N-3 in thymine to C-4 changes the keto group at C-4 to an enol group and alters the base pairing so that thymine can pair with guanine instead of adenine. Likewise, a keto-enol shift can occur in guanine, N-1 hydrogen moving to C-6 oxygen (this also permits guanine to pair with thymine but only when the latter is in the keto form). Similar shifts of hydrogen to and from nitrogen atoms (amino-imino shift) can occur, creating various other mispairing arrangements. However, such tautomeric shifts, as well as the ionizations of protons at positions 1 and 3 of guanine and thymine, respectively, occur to such a small extent under usual physiologic conditions that mispairing is infrequent. Nevertheless, such rare structures provide a strong theoretic basis for nucleotide (base) substitution and mutation.

Obvious possibilities in base substitution include the substitution of

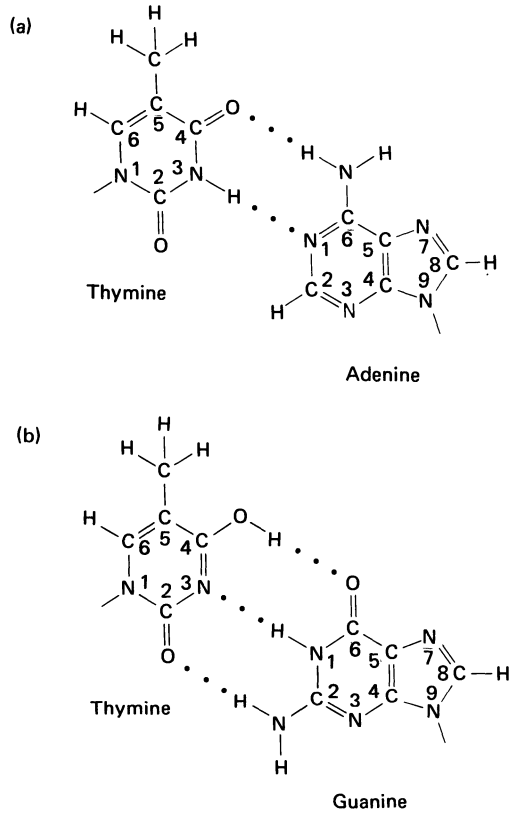


Fig. 43. Normal and abnormal base-pairing of thymine.

one purine for another or one pyrimidine for another. Such substitutions are called transitions and these may be contrasted with the possibility of purines substituting for pyrimidines or vice versa, which are called transversions. Each may be summarized in terms of the common purines and pyrimidines of DNA and RNA as follows:

Transitions: $C \rightleftharpoons T$ or U, and $A \rightleftharpoons G$
Transversions: $A \rightleftharpoons C \rightleftharpoons G \rightleftharpoons U$ or T

Spontaneous mutations of viruses can be explained on the basis of one or another of the above molecular mechanisms (see Drake 1970; Stent 1971; Freese 1971). However, evidence that a particular mechanism actually operates is more convincing for some of the chemically induced mutations than it is for the spontaneous ones.

Transitions appear to be caused by most of the major classes of mutagenic chemicals: nitrous acid, base analogs, hydroxylamine, and alkylating

agents. Additions and deletions, resulting in frame-shift mutations, are induced by intercalating chemicals such as acridine and various amino acridines. It appears that some alkylating agents, notably ethylethane sulfonate, may cause transversion. Transitions and transversions affecting as they do one nucleotide are said to cause point mutations.

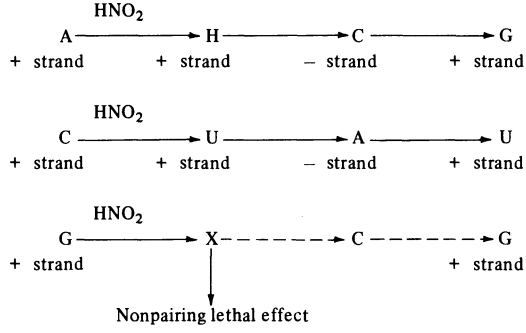
The type of mutational alteration in a viral nucleic acid can tentatively be identified by genetic criteria briefly summarized as follows: Transitions are induced to revert with base analogs; frame-shift mutations are generally induced to revert by treatment with acridines; and mutants not induced to revert with either type of agent but that do revert spontaneously are likely to be transversions.

Before considering individual mutagens some distinction should be made between *in vitro* and *in vivo* mutagenic action. In some cases, a viral nucleic acid, either isolated or in the virion, can be altered by chemical treatment in such a way as to represent a mutation or to induce a mutation during subsequent replication. This is *in vitro* mutagenesis; some chemicals causing mutation in this manner are nitrous acid, hydroxylamine, and alkylating agents. *In vivo* mutagenesis occurs when the mutagenic agent is present during replication of the viral nucleic acid; base analogs and intercalating chemicals can act mutagenically at this stage of virus replication.

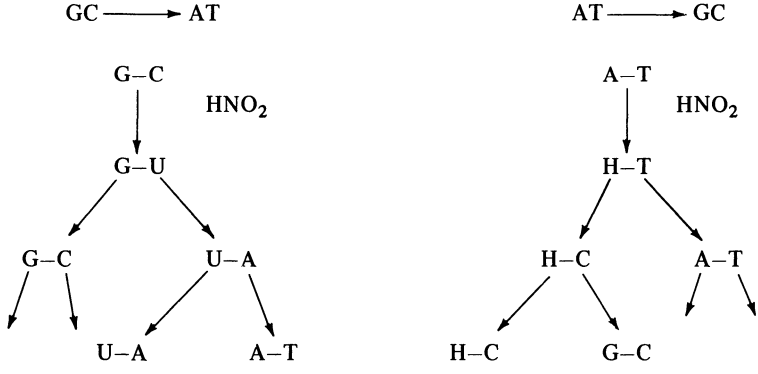
a. Nitrous Acid

Nitrous acid is one of the most effective mutagenic chemicals. As indicated in Sect. VIA, 3c, it oxidatively deaminates primary amino groups of purines and pyrimidines in viral nucleic acids. Thus adenine (A) is converted to hypoxanthine (H), cytosine (C) to uracil (U), guanine (G) to xanthine (X). The C to U transition represents a direct substitution of one common nucleotide for another; the other two nitrous acid-induced transitions yield natural products, but ones seldom, if ever, found in nucleic acids. Upon further replication the transition of cytosine to uracil is perpetuated while the hypoxanthine resulting from deamination of adenine pairs with cytosine, which, in turn, pairs with guanine so that ultimately an adenine is replaced by guanine. Both these transitions are potentially mutagenic (they are also potentially inactivating as previously noted). Xanthine, produced by the deamination of guanine can pair with cytosine, which in the next replicative step will pair with guanine thus restoring the original sequence. However, xanthine appears to ionize more at pH values around neutrality than other bases, and this interferes with its base-pairing function producing a lethal effect. This lethality is demonstrated in a practical manner by the observation that deamination of guanine-containing polynucleotides renders them inactive as templates (Basilio et al. 1962). The direct and indirect effects of nitrous acid may be sketched for RNA and DNA showing just the relevant base or bases in the polynucleotide chain as follows:

Replicative events after oxidative deamination of bases in single-stranded RNA:



The same scheme applies to the appropriate bases in double-stranded RNA. Replicative events after oxidative deamination of bases in double-stranded DNA:



The guanine to xanthine transition in DNA proceeds as indicated for RNA except that complementary bases are carried along in the scheme.

Nitrous acid has been the most effective mutagen for RNA but relatively poor for DNA, presumably because amino groups are virtually unavailable in double-stranded nucleic acid molecules except in zones of incipient denaturation (strand separation). Instead of deamination, several inactivating side reactions tend to occur with DNA as indicated in Sec. VIA, 3c.

The interaction between proteins and nucleic acid can affect the mutagenicity of nitrous acid as shown by difference in reactivity of free and virus-incorporated RNA of TMV. Under conditions that result in considerable deamination of adenine, guanine, and cytosine in isolated TMV-RNA, essentially no deamination of guanine occurs in whole virus while 20 and

30 percent of the adenine and cytosine, respectively, are deaminated (Schuster and Wilhelm 1963). A consequence of this difference in reactivity is that the inactivating effect of nitrous acid on guanine is reduced in relationship to the mutagenic action on cytosine and adenine (Singer and Fraenkel-Conrat 1969).

Some relationships of nitrous acid mutagenesis to the genetic code will be considered in the next section.

b. Hydroxylamine

Hydroxylamine is considered one of the most specific *in vitro* mutagens since it and its derivatives (for example, methoxyamine and *N*-methylhydroxylamine) react only with pyrimidines; the reaction with cytosine (or 5-hydroxymethylcytosine in T-even phages) is very probably mutagenic while that with uracil is inactivating (Drake 1970). As indicated earlier, the reaction with uracil (and presumably with thymine also) occurs mainly at pH 9–10, whereas the reaction with cytosine takes place optimally around pH 6. The reaction shown at the left in Sec. VIA, 3e, involving the 4-amino group of cytosine, is considered to be mutagenic (Singer and Fraenkel-Conrat 1969; Phillips and Brown 1967). The base pairing of cytosine is altered by formation of the hydroxylamine derivative so that it can pair with adenine in place of the usual guanine; upon further replication (when DNA is involved) this results in an A-T pair in place of G-C, thymine having taken the place occupied by cytosine in the sequence of the original polynucleotide chain (Freese 1971).

In vivo mutagenic action on the single-stranded DNA phages S13 and ØX174 by hydroxylamine has been reported (Tessman et al. 1965). Transitions affecting all of the bases appeared to be effected when bacterial cells were treated with hydroxylamine prior to infection. The precise mechanism of this nonspecific *in vivo* induction of mutants is unclear.

c. Alkylating Agents

Alkylation is effected by treatment of viruses or their nucleic acids with nitrogen and sulfur mustards, alkyl sulfates, alkyl sulfonates, ethylene oxide, propylene oxide, and so on. The alkyl group is introduced at the 7 position of guanine and to some extent elsewhere such as the N-1 of adenine (the latter may be the main reaction with denatured or single-stranded DNA, while the former seems to be the major reaction with double-stranded DNA). The alkylation of guanine causes a tautomeric shift and a change in base pairing, resulting mainly in transitions from G-C to A-T pairs in DNA (Freese 1971).

d. Base Analogs

Base analogs are *in vivo* mutagens that cause mutations during replication of DNA, as was first demonstrated by the classic study of Litman and

Pardee (1956) with T2 coliphage and 5-bromouracil. While there is little doubt that some base analogs (for example, 2-thiouracil and 8-azaguanine) inactivate plant viruses when incorporated into the viral RNA, it is uncertain that any of them is mutagenic. Not many base analogs are mutagenic with DNA either, the halogenated uracils (5-bromo, 5-chloro, and 5-iodouracil) and 2-amino purine being most efficient in this regard, especially when introduced to the cells in the form of their respective deoxynucleosides (Freese 1971). The halogenated uracils are apparently incorporated into DNA in place of thymine, bromouracil (BU) working especially well presumably because the 5-bromo group occupies about the same space as the 5-methyl group of thymine. However, the electronic characteristics of Br and CH_3 are quite different so that a certain tautomeric shift occurs more frequently with BU than with thymine. Thus, BU, like thymine, is normally in the keto form (C=O group at position 4) in which it, like thymine, pairs with adenine; a tautomeric shift of H from N-3 to the O of the keto group at position 4 converts the keto into an enol group (C-OH) and promotes pairing with guanine instead of with adenine.

This mispairing can cause a transition in either of two ways: If BU is in the rare enol form during synthesis of a DNA strand, it can be incorporated opposite guanine in place of cytosine (error due to substrate enol, also called "mistake in incorporation" or "substrate transition"). When BU has been incorporated into a DNA strand and that strand serves as a template for replication, adenine is normally paired with BU (keto form), but if BU is in the rare enol form, it will pair with guanine instead (error due to template enol, also called "mistake in replication" or "template transition"). Thus BU causes *in vivo* transitions: GC to AT and AT to GC. The GC to AT transition results as shown in Figure 44a when BU is in the enol form at the time of incorporation (step 2 in the figure) while the AT to GC transition occurs during replication when BU, having been incorporated while in the common keto form (Figure 44b, step 2) shifts to the enol form and pairs with guanine (Figure 44b, step 3).

Comparison of a and b in Figure 44 suggests that BU-induced transitions should be reversible by treatment with BU; such reversions have been observed experimentally (Freese 1971).

e. Intercalating Chemicals

Certain acridine dyes and the structurally similar phenanthridines can intercalate between adjacent purine and pyrimidine base pairs of the DNA double helix (Lerman 1964; Cairns 1962). Examples of these two classes of intercalating molecules are shown in Figure 45. Intercalation of a molecule such as proflavin into a phage DNA in infected bacterial cells can cause *in vivo* mutations that appear based on additions or deletions usually of one or a few nucleotides (Lerman 1964; Drake 1970). Since transcription of the

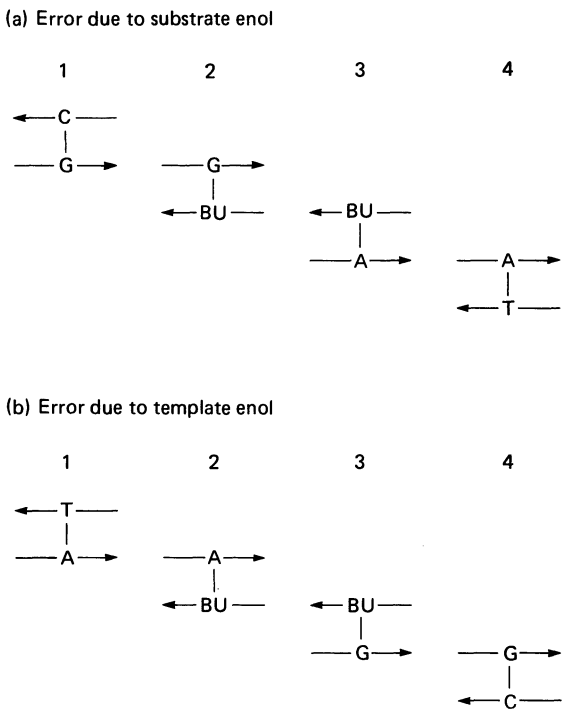
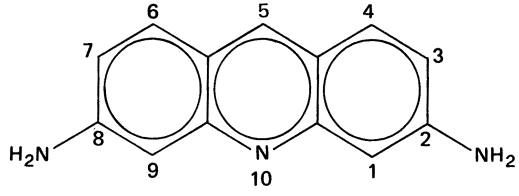


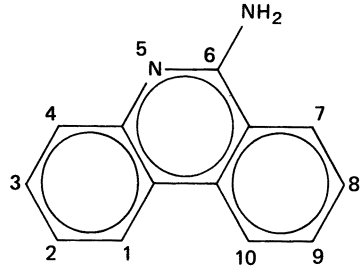
Fig. 44. Transitions caused by bromouracil.

genetic code appears to begin at a fixed point and to move along a linear series of nonoverlapping, nucleotide triplets, addition or deletion of even a single nucleotide can have a profound effect on the transcription of a DNA strand. The transcribing is shifted so that every codon (nucleotide triplet) to the right of the addition or deletion is altered. Mutants resulting from such altered transcription are called frame-shift or phase-shift mutants because the frame of reference for transcribing the nucleotides as triplets has been shifted; in other words, the transcription has been shifted out of phase. Conceptually at least, the addition or deletion of nucleotides in the form of one or more codons might be far less drastic than insertion or deletion of a single nucleotide because frame shifts would be avoided.

However, the key question of how intercalating compounds accomplish their mutagenic action is not yet clear. It is thought that single-strand breaks ("nicks") occur quite frequently in DNAs, and Streisinger et al. (1966) surmised that intercalated molecules stabilize the mispairing that may occur between complementary strands of DNA that have undergone breakage and partial strand separation. Mispairing is accompanied by looping out of one of the strands of the duplex, with or without some excision by



Proflavin (2,8-diaminoacridine)



6-aminophenanthridine

Fig. 45. Basic formulas for two classes of intercalating chemicals that induce frame shifts in DNA.

nuclease, and is followed by repair, which leaves one strand with its original composition while the other is either lengthened or shortened. These steps are illustrated in Figure 46.

The reversion of mutations caused by acridines and similar compounds, which is also induced by treatment with acridines, presumably results from restoration of the proper number of base pairs by addition or deletion. [If hundreds of nucleotides have been deleted, as occasionally occurs (Benzer 1961), restoration would not be expected.] The reverting addition or deletion need not be at the site of the original alteration but can be at a closely linked site. On the basis of revertibility by acridines, it appears that the majority of spontaneous mutants of T4 phage are frame shift (Drake 1970).

The mutagenicity of acridines can be complicated by such factors as the different permeabilities of cells to various acridine derivatives and the presence or absence of visible light since acridines can also induce mutations photodynamically. The latter type of mutation appears to involve nucleotide substitution rather than addition-deletion. It has been observed with phage T4 (Ritchie 1964, 1965).

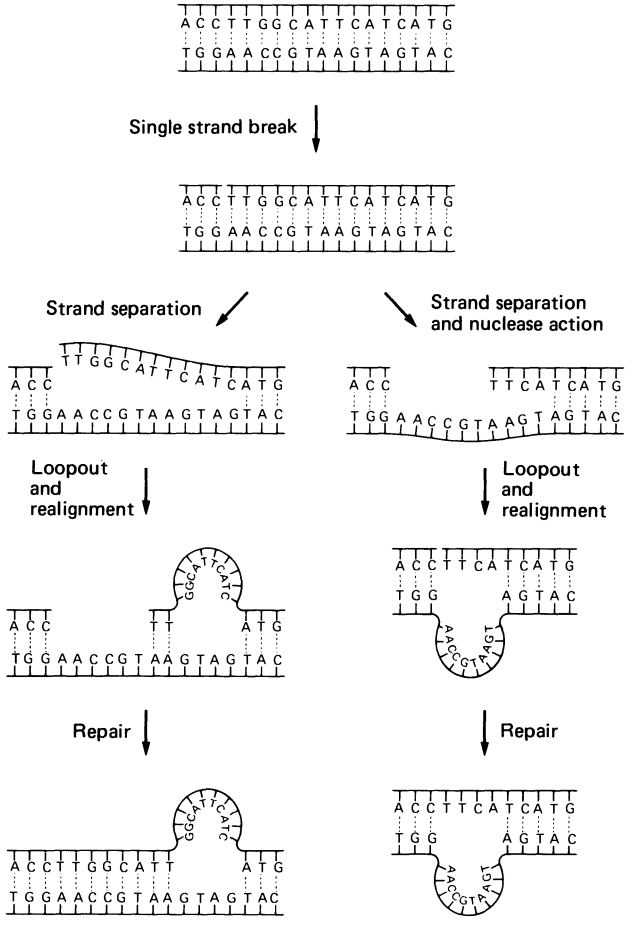


Fig. 46. Hypothetical mechanism for the production of frame-shift mutants according to the Streisinger model. As shown, after a single break has been introduced in one of the strands of DNA, strand separation occurs over a small distance, either followed by nuclease action on the open strand (*right*) or not (*left*). In both cases, loopout of a strand occurs next but in different places, and is followed by repair that closes the interrupted strand. In the next round of replication, it is evident that the top strand of the product on the left will give rise to a duplex containing an addition, whereas the top strand of the product on the right will yield a duplex with a deletion. (Adaptation Knight 1974 from Streisinger et al. 1966.)

2. *Effect of Mutations on Viral Proteins*

Mutation is based on significant changes in the composition of nucleic acids, but such alterations are directly demonstrable with great difficulty if at all by current analytic techniques. Even in a case where the nature of the chemical change induced by a mutagenic chemical is well established, such as the oxidative deamination of cytosine to uracil, the number of cytosines that must be converted to uracil in order to be detectable by analysis is many times larger than theoretically required for mutagenesis, although small changes might conceivably be detected by sequencing techniques described earlier. Consequently, mutation often has been analyzed by genetic methods which, while very valuable, are by nature indirect and highly deductive. However, since mutations are generally reflected in the structure of some protein (the sequence of amino acids is determined by the sequence of nucleotides in the appropriate gene), the consequences and mechanisms of mutation can also be evaluated by analysis and sequencing of proteins. This is technically feasible in at least some cases. A few examples will be cited here to illustrate the consequences of spontaneous mutation and of the mutagenic procedures sketched in the previous section.

Strains of tobacco mosaic virus are exceedingly numerous and varied in their properties. Furthermore, most strains are easily grown in tobacco plants and can be isolated and readily purified in sufficient quantities for analysis.

Analyses of the coat proteins of some spontaneous mutants of TMV were made many years ago (Knight and Stanley 1941; Knight 1942, 1943, 1947b). The complete analyses of strain proteins (Knight 1947b) were made by microbiological assay at a time when the only other complete analysis of any protein was that of Brand et al. (1945) of beta-lactoglobulin. Now, complete amino acid analyses of proteins are made routinely and with greater accuracy with automatic analyzers employing ion exchange columns (see Sec. IIIA, 2a). Scores of proteins have been analyzed (Dayhoff 1972, 1973), including numerous viral proteins. Nevertheless, the following conclusions drawn from the microbiological assays of the TMV strain proteins have been confirmed and strengthened by subsequent more refined analyses: (1) Strains often differ from one another in protein composition. These differences usually appear in proportions of the constituent amino acids. However, in some cases, certain amino acids (notably histidine and methionine) may be present in one strain which are completely lacking in another. (2) The closely related strains, which presumably have arisen by one or few mutational steps, are very similar in protein composition. In fact, there seem to be no differences at all in the proteins of common TMV and M. (3) Differences in strain proteins seem to involve almost any of the constituent amino acids.

These conclusions are consistent with current genetic theory, pertinent portions of which are as follows: The genetic material of any virus is its nucleic acid whose linear sequence of nucleotides comprise a few to many genes (also called cistrons), depending mainly on the size of the nucleic acid. One of these genes determines the composition and sequences of amino acids of a viral coat protein. Spontaneous mutation is essentially a random process that may or may not affect the coat protein gene, and if it does might be expected to alter randomly any one of the amino acids in the coat protein. Closely related strains would be expected to have fewer differences in coat protein than distantly related strains.

These principles are illustrated especially well by comparing the sequences of amino acids in viral proteins of related groups of viruses. A graphic way to express the results is not to present the detailed sequential information but rather to express the results in terms of the percent difference between sequences of the viral proteins. This is done in Tables 42 and 43 for the coat proteins of some strains of tobacco mosaic virus and of some small RNA phages, respectively. The viruses in each of these two groups are considered related because they exhibit common physical, chemical, and serological characteristics, as well as other similarities, such as host specificity, mode of infection, and so forth. The values shown in Tables 42 and 43 illustrate the point made earlier, that viral coat proteins of different strains of a virus may not differ in any respect (hence their coat protein genes must be identical) or they may differ somewhat or considerably, again reflecting various degrees of similarity in their coat protein genes.

The determination of the genetic code has made it possible to relate viral protein analyses to nucleic acid sequences (that is, to relate a gene

Table 42. Comparison of Amino Acid Sequences of Some Spontaneous Mutants of Tobacco Mosaic Virus.[a]

	TMV	OM	Dahl.	ORS	U2	HR
Strain	% Difference Between Sequences					
TMV (common tobacco strain)	0	2	18	26	27	55
OM (tobacco strain)	2	0	18	26	27	56
Dahlemense (tobacco strain)	18	18	0	27	30	54
ORS (orchid strain)	26	26	27	0	30	53
U2 (tobacco strain)	27	27	30	30	0	55
HR (ribgrass strain)	55	56	54	53	55	0

[a]Adapted from Dayhoff 1972.

Table 43. Comparison of Amino Acid Sequences of Some Related RNA-Containing Bacteriophages.[a]

	f2	MS2, R17	ZR	fr	Qβ
Bacteriophage	% difference between sequences				
F2	0	1	1	17	75
MS2, R17	1	0	0	16	75
ZR	1	0	0	16	75
fr	17	16	16	0	76
Qβ	75	75	75	76	0

[a]Adapted from Dayhoff 1972 but not corrected to reflect later analytical data showing three differences between the coat proteins of MS2 and R17 (Min Jou et al. 1972), which would also alter slightly the other values in the table.

product to its gene on the molecular level). In fact, during early stages of development of the genetic code, analyses of nitrous acid mutants of TMV were helpful in confirming codons as well as in testing the universality of the code (Ochoa 1963). Additional confirmation of the code has come from later extensive studies of the coat proteins of the f2-MS2-R17 group of RNA phages (see Min Jou et al. 1972).

The genetic code presented in Table 44 has developed over the course of about ten years. It was proposed by Crick et al. (1961), on the basis of several lines of evidence, that the genetic code is a triplet, degenerate (degenerate means that more than one triplet codes for a given amino acid), nonoverlapping code which is read from a fixed starting point. These premises have been validated over the years. The 64 triplets of the genetic code are shown in Table 44 using A, G, C, and U as abbreviations for the ribonucleotides: adenylic acid, guanylic acid, cytidylic acid, and uridylic acid, respectively.

The earliest assignments of nucleotides to the codons (codons are the coding triplets) were derived from data obtained in cell-free syntheses of polypeptides from radioactive amino acids using synthetic polyribonucleotides of differing compositions as messengers (Matthaei and Nirenberg 1961; Nirenberg and Matthaei 1961; Nirenberg et al. 1962). The essential components of this system were washed *E. coli* ribosomes and tRNAs, an ATP-generating system, labeled amino acid, and polynucleotides containing different proportions of nucleotides as test messengers. By noting the amount of incorporation of a given ^{14}C-labeled amino acid in response to enzymatically synthesized messengers containing different proportions of nucleotides, it was possible to deduce more than a third of the codons (Martin et al. 1962; Lengyel et al. 1961; Speyer et al. 1962a, 1962b; Lengyel et al. 1962; Matthaei et al. 1962). Later, synthetic messengers whose nucleotide sequences were more precisely known were prepared and used by

Table 44. The Genetic Code.

Ala	GCA	Gly	GGA	Pro	CCA
	GCC		GGC		CCC
	GCG		GGG		CCG
	GCU		GGU		CCU
Arg	AGA	His	CAC	Ser	AGC
	AGG		CAU		AGU
	CGA				UCA
	CGC	Ile	AUA		UCC
	CGG		AUC		UCG
	CGU		AUU		UCU
Asp	GAC	Leu	CUA	Thr	ACA
	GAU		CUC		ACC
			CUG		ACG
Asn	AAC		CUU		ACU
	AAU		UUA		
			UUG	Trp	UGG
Cys	UGA				
	UGC				
	UGU	Lys	AAA	Tyr	UAC
			AAG		UAU
Glu	GAA				
	GAG	Met	AUG	Val	GUA
		FMet			GUC
Gln	CAA	Phe	UUC		GUG
	CAG		UUU		GUU

Initiation codons: AUG; GUG.
Termination codons: amber UAG, ochre UAA, umber (opal) UGA.

Khorana and associates (1966) to test the specificities of various codons in cell-free syntheses. Still another ingenious method for testing codons was developed by Nirenberg and associates (1966). This technique was based on the synthesis of all 64 triplets and the demonstration that these would bind to ribosomes where in turn specific ^{14}C-amino acid tRNAs would attach while the nonspecific ones would not; the codon-ribosome-amino acid-tRNA complex was retained on a fine cellulose nitrate filter and the quantity could be ascertained by radioactivity measurements in a scintillation counter. If the codon was not right, the amino acid-tRNA would pass through the filter and subsequent radioactivity counts would be low.

All of the procedures sketched above plus other lines of evidence have contributed to the codon catalog presented in Table 44. Each of the procedures used to identify the codons showed some anomalies, but together generally support the listing given.

The arrangement used in Table 44 was developed from the data of P.

Leder in Sober (1970) and presented in Knight (1974). Some advantages of this unconventional arrangement are (1) the amino acids are listed in alphabetical order for quick reference; (2) all of the codons associated with a given amino acid are present in one place and are in alphabetical order; (3) some basic features of the genetic code are immediately apparent; for example, the general identity of the first two nucleotides and the variability of the third in the codons for a particular amino acid.

With the genetic code in mind and considering molecular mechanisms of mutation, the question could be raised concerning the origin of spontaneous mutants. This is a question that cannot yet be answered definitively. However, largely on the basis of what mutagenic agents can cause reversions in the T-even bacteriophages, it appears that spontaneous mutations of these viruses may involve mainly frame shifts rather than base substitutions (Brenner et al. 1961). In the case of plant viruses a comparison of the sequences of amino acids in the coat proteins of the common and HR strains of tobacco mosaic virus (Funatsu and Funatsu 1968; Hennig and Wittmann 1972) suggests that if HR evolved from TMV a rich variety of mutational events must have occurred, including transitions, transversions, frame shifts, and deletions. Further study is needed to define better the mutational mechanisms operative in nature.

The laboratory-produced mutants of the tobacco mosaic virus series have illuminated a number of relationships between genes and gene products. Some of the findings will be briefly reviewed here.

Nitrous acid has proved to be the most efficient mutagen for strains of TMV. The coat proteins of scores of these mutants were analyzed (Funatsu and Fraenkel-Conrat 1964; Henning and Wittmann 1972). In a large number of cases, the coat protein gene of the virus was unaffected as shown by the lack of any differences in coat protein. Where changes in coat protein were found in the nitrous acid mutants, most of them proved to be transitions of the A to G and C to U types expected in nitrous acid mutagenesis. This is illustrated by some data compiled in Table 45, which provide support for operation of the genetic code of Table 44 and are consistent with the idea of a universal genetic code. Although not indicated in Table 45, most of the nitrous acid mutants showing any amino acid exchange showed only one. This can be taken as evidence for a non-overlapping genetic code, since one change in an overlapping code would often have brought about two exchanges of amino acids next to each other in the polypeptide chain. No such contiguous exchanges were observed.

The nature of the code as presented in Table 44 suggests several potential effects of mutagenesis on the gene products of a viral nucleic acid:

1. If a base substitution occurs in the first or second nucleotide of a triplet, a different amino acid will almost always result from translation of the codon (arginine and leucine are partial exceptions); when the substitu-

Table 45. Amino Acid Exchanges Found and Corresponding Codon Changes Associated with Coat-Protein Mutants Obtained by Treatment of Tobacco Mosaic Virus with Nitrous Acid.[a]

No. of Mutants	Amino Acid Exchange	Corresponding Codon Change	Nature of Change
1	Arg → Cys	CGU → UGU	C → U
4	Arg → Gly	AGG → GGG	A → G
1	Arg → Lys	AGA → AAA	A → G
4	Asn → Ser	AAU → AGU	A → G
1	Asp → Gly	GAU → GGU	A → G
1	Gln → Arg	CAG → CGG	A → G
1	Glu → Asp	GAA → GAU	A → U
2	Glu → Gly	GAA → GGA	A → G
1	Ile → Met	AUA → AUG	A → G
5	Ile → Val	AUU → GUU	A → G
4	Pro → Leu	CCU → CUU	C → U
2	Pro → Ser	CCC → UCC	C → U
1	Ser → His	UCU → CAU	UC → CA
3	Ser → Leu	UCG → UUG	C → U
5	Ser → Phe	UCU → UUU	C → U
2	Thr → Ala	ACU → GCU	A → G
6	Thr → Ile	ACU → AUU	C → U
2	Thr → Met	ACG → AUG	C → U
1	Tyr → Cys	UAC → UGC	A → G
1	Val → Met	GUG → AUG	G → A

[a]Compiled from Hennig and Wittmann 1972.

tion occurs in the third nucleotide, no amino acid change will result if the amino acid concerned is one coded for by four triplets. But there may or may not be a change if the amino acid has less than four codons, depending on the base substituted. In addition there is a special case: a third-nucleotide transition in the Trp codon produces the umber peptide chain termination codon.

2. Termination codons can be generated by single transitions of Gln and Trp codons or single transversions of Glu and Lys codons.

3. Base substitution in a single nucleotide of a codon can lead to any of the following effects on a protein: polypeptide chain termination in a few cases (see point 2); no change in amino acid and hence no effect (many cases, see point 1); a change in amino acid either with or without detectable effect on protein function.

4. A frame shift has potentially the most drastic effect on the genetic function of nucleic acid because it can produce any of the consequences mentioned above, and in generous measure, since by nature it almost surely affects more than one codon.

Mutations that cause chain termination and hence loss of function of some protein are invaluable in genetic studies (Hayes 1968), especially when combined with the observation that a permissive host may be available that can respond not by chain termination but rather by translation of the termination codon as an amino acid codon. The latter response is known as suppressor effect and is based on a suppressor gene in the permissive host that appears to code for a minor species of amino acid-tRNA whose anticodon (triplet complementary to a codon) is complementary to the termination codon (Garen 1968). Thus a functional polypeptide is produced in the presence of a suppressor; for example serine may be inserted in the permissive host in response to the amber codon (Tooze and Weber 1967).

Terminations resulting from the amber codon (UAG) have been detected frequently among phage mutants and are termed amber (am) mutants (Sarabhai et al. 1964; Hayes 1968, p. 484). Amber mutants are distinguished from ochre or umber mutants by their responses to different suppressors, whose specificities were established by mutagenesis and protein analytic studies on alkaline phosphatase of *E. coli* (Garen 1968).

A series of amber mutants of the small RNA phages, f2, R17, and MS2, were produced mostly by treatment with nitrous acid but also by hydroxylamine and fluorouracil mutagenesis (Zinder and Cooper 1964; Webster et al. 1967; Gussin 1966; Tooze and Weber 1967; Min Jou et al. 1972). Some of these mutations were in the coat protein gene and were located by amino acid sequence analyses. All of them involved glutamine, except one, which affected tryptophan. These changes provide chemical confirmation for the amber nature of the mutation since the amber codon (UAG) could predictably arise by the treatments employed from the glutamine and tryptophan codons, CAG and UGG, by simple transitions.

A neat demonstration of the colinearity of gene nucleic acid and gene product (protein) was made with amber mutants of coliphage T4 by Sarabhai et al. (1964). The experiment devised was based on the concept that if there is colinearity between gene and protein, each of several different amber mutants should yield after transcription and translation a polypeptide chain whose length would depend on the distance of mutation from one end of the gene. By infecting separate cultures of nonpermissive bacteria with different amber mutants (grown up in the permissive host) and adding radioactive amino acids late in the infectious process when phage head protein is predominately synthesized, it was possible to identify a series of proteins that by comparison with wild type protein did represent polypeptides of different lengths.

As suggested earlier, mutation can change the function of a protein—whether it be plant virus coat protein, influenza hemagglutinin, or phage lysozyme. There are numerous cases of such effects but a few examples will illustrate some of the possibilities.

Some of the nitrous acid mutants of tobacco mosaic virus are difficult to pass in series, which was found to be related in certain cases to the defective nature of the coat protein of the mutant virus (Siegel and Zaitlin 1965). Such protein may be unable to assemble around the viral RNA; consequently the RNA is readily digested by plant ribonucleases when attempts are made to isolate or transfer the virus. Analysis of the protein of one of these mutants, PM2, revealed only two differences between it and wild type TMV protein: threonine in position 28 had been replaced by isoleucine, and glutamic acid in position 95 had been replaced by aspartic acid (see Table 13 for sequence of amino acids in TMV protein). The pronounced effect of these changes on the ability of the protein to aggregate into tight, rodlike (tubular) structures is illustrated in the electron micrographs of Figure 47 in which polymerized wild type protein and polymerized PM2 protein are contrasted. It is not known whether both of the amino acid exchanges noted above contribute to the abnormal aggregation of PM2 protein, but it has been suggested that the crucial exchange might be the one substituting leucine for threonine since this amounts to

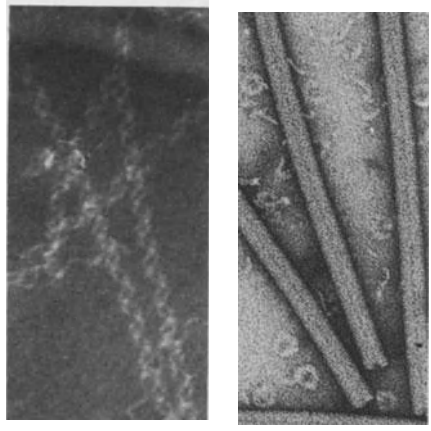


Fig. 47. Electron micrographs, prepared by the negative staining technique, of elongated structures formed in the absence of nucleic acid by aggregation of tobacco mosaic virus coat protein. *a*. Open helical structures formed by aggregation of protein of TMV nitrous acid mutant PM2. *b*. Regular close-packed helical structures formed by aggregation of TMV protein. (*a*, courtesy M. Zaitlin; *b*, courtesy R. C. Williams.)

replacing an amino acid residue having a hydrophilic side chain (Thr) with one of hydrophobic character (Leu), whereas the amino acids in the other exchange are essentially equivalent.

Two nitrous acid mutants of TMV isolated by Sengbusch and Wittmann (1965) presented an unusual opportunity to evaluate the effect on serological specificity of single amino acid exchanges in a viral coat protein. Differences in serological specificity had previously been demonstrated between TMV and TMV from all of whose protein subunits the C-terminal threonine residues had been removed (Knight 1961), but the unique feature of the two nitrous acid mutants (Ni 118 and Ni 1927) was that they exhibit the same amino acid exchange, proline to leucine, but in different positions in the polypeptide chain. The amino acid exchange occurred in position 20 (near the N-terminal) in Ni 118 and in position 156 (near the C-terminal; see Table 13) in Ni 1927. In serologic tests it was found that the mutant with the exchange at position 156 could be distinguished from TMV but the other mutant could not. From earlier studies it was known that the C-terminal of the TMV protein subunits are located near the surface of the virus particle, whereas the N-terminal is folded in toward the interior of the particle. Hence, the single exchange located near the surface of the particle in Ni 1927 registered serologically while the same exchange internally oriented in Ni 118 was serologically undetectable.

One of the virus-specific proteins of the T-even phages is the enzyme, lysozyme. This enzyme is a product of the e gene (endolysin gene) of phage T4 and can be readily purified and analyzed. It is possible to produce mutants of T4 in which the phage lysozyme is affected, including mutants resulting from *in vivo* mutagenesis with acridines (Streisinger et al. 1966). It will be recalled from the earlier discussion of frame-shift mutants that acridines such as proflavin cause deletions or additions of nucleotides (often one nucleotide) resulting in gross changes in the affected gene. However, if the deletion of a nucleotide is followed by addition of one beyond the region of mutation (or the reverse, addition followed by deletion), the damage can sometimes be corrected. Terzaghi and associates (1966), working with proflavin mutants of T4 phage, obtained a double mutant whose lysozyme differed from that of wild type in that it was partially rather than fully active and possessed a different sequence of five amino acids in an eight amino acid sequence:

Wild type: -Thr-Lys-Ser-Pro-Ser-Leu-Asn-Ala-
Mutant : -Thr-Lys-Val-His-His-Leu-Met-Ala-

The appropriate codons of the mRNA for the eight-amino acid segment of wild type lysozyme can be shown as follows:

1 4 7 10 13 16 19 22
A C A - A A A - A G U - C C A - U C A - C U U - A A U - G C U -

Deletion-addition events explaining the observed amino acid sequence of the double mutant lysozyme are as follows: Delete nucleotide 3 (A), shift nucleotides beyond 3 one place to the left to form new triplets; add G between nucleotides 22 and 23. The resulting mRNA segment is

```
1       4       7       10      13      16      19      22
A C A - A A A - G U C - C A U - C A C - U U A - A U G - G C U -
```

In addition to confirming postulates about the nature of frame-shift mutations, the results of these studies also confirmed the concept that mRNA is translated in the 5′ to 3′ direction.

In relating a gene to its product, the ultimate achievement on the molecular level is to make a direct comparison of the relevant mRNA nucleotide sequences and the amino acid sequences of the protein specified. Such a comparison has been made with coliphage MS2 (Min Jou et al. 1972). This virus is a small RNA phage with only three genes—one for an RNA replicase (also called RNA polymerase or synthetase), one for the main coat protein, and a third coding for a special coat protein called A protein or maturation protein. The main coat protein of this phage, a 129-amino acid polypeptide, can be readily isolated and its amino acid sequence had been determined, although with three errors according to the gene analysis referred to here. The coat protein gene was isolated and analyzed as follows.

^{32}P-labeled MS2 RNA was subjected to limited digestion with ribonuclease T_1 at low temperatures which yielded fragments that could be separated on neutral polyacrylamide gels and were small enough for direct sequence analysis by procedures described in Sec. IIIB, 2. Knowledge of the amino acid sequences of MS2 coat protein, coupled with the genetic code, enabled screening of the oligonucleotides obtained from ribonuclease T_1 digestion for the ones related to coat protein gene. This resulted in isolation and sequencing of five fragments spread over the gene (Min Jou et al. 1971). These and many other analyses were combined by taking advantage of overlapping oligonucleotides and knowledge of what the order of nucleotides should be from the amino acid sequence of the protein. The nucleotide sequence obtained is shown in Figure 48. It includes not only the coat protein gene with initiation and termination codons but also the ribosomal binding site for the coat protein gene and one for the RNA polymerase together with the initiation codon (AUG) and the first six codons for the polymerase.

The nucleotide triplets which were found to code for the 129 amino acids of the MS2 coat protein are indicated in Table 46, the figures in parentheses indicating the number of times a particular codon is used in the gene. Forty-nine different codons are employed and there is no clear basis yet for explaining the choice between degenerate codons. However, it will be noted that all of the codons found agree with standard ones listed in the codon catalog.

														. . . (G)·	AUA·	GAG·	CCC·	UCA·	ACC·	GGA·	GUU·	UGA·	AGC·	AUG·
GCU·	UCU·	AAC·	UUU·	ACU·	CAG·	UUC·	GUU·	CUC·	GUC·	GAC·	AAU·	GGC·	GGA·	ACU·	GGC·	GAC·	GUG·	ACU·	GUC·	GCC·	CCA·	AGC·	AAC·	UUC·
Ala	Ser	Asn	Phe	Thr	Gln	Phe	Val	Leu	Val	Asp	Asn	Gly	Gly	Thr	Gly	Asp	Val	Thr	Val	Ala	Pro	Ser	Asn	Phe
1				5					10					15					20					25
GCU·	AAC·	GGG·	GUC·	GCU·	GAA·	UGG·	AUC·	AGC·	UCU·	AAC·	UCG·	CGU·	UCA·	CAG·	GCU·	UAC·	AAA·	GUA·	ACC·	UGU·	AGC·	GUU·	CGU·	CAG·
Ala	Asn	Gly	Val	Ala	Glu	Trp	Ile	Ser	Ser	Asn	Ser	Arg	Ser	Gln	Ala	Tyr	Lys	Val	Thr	Cys	Ser	Val	Arg	Gln
				30					35					40					45					50
AGC·	UCU·	GCG·	CAG·	AAU·	CGC·	AAA·	UAC·	ACC·	AUC·	AAA·	GUC·	GAG·	GUG·	CCU·	AAA·	GUG·	GCA·	ACC·	CAG·	ACU·	GUU·	GGU·	GGU·	GUA·
Ser	Ser	Ala	Gln	Asn	Arg	Lys	Tyr	Thr	Ile	Lys	Val	Glu	Val	Pro	Lys	Val	Ala	Thr	Gln	Thr	Val	Gly	Gly	Val
				55					60					65					70					75
GAG·	CUU·	CCU·	GUA·	GCC·	GCA·	UGG·	CGU·	UCG·	UAC·	UUA·	AAU·	AUG·	GAA·	CUA·	ACC·	AUU·	CCA·	AUU·	UUC·	GCU·	ACG·	AAU·	UCC·	GAC·
Glu	Leu	Pro	Val	Ala	Ala	Trp	Arg	Ser	Tyr	Leu	Asn	Met	Glu	Leu	Thr	Ile	Pro	Ile	Phe	Ala	Thr	Asn	Ser	Asp
				80					85					90					95					100
UGC·	GAG·	CUU·	AUU·	GUU·	AAG·	GCA·	AUG·	CAA·	GGU·	CUC·	CUA·	AAA·	GAU·	GGA·	AAC·	CCG·	AUU·	CCC·	UCA·	GCA·	AUC·	GCA·	GCA·	AAC·
Cys	Glu	Leu	Ile	Val	Lys	Ala	Met	Gln	Gly	Leu	Leu	Lys	Asp	Gly	Asn	Pro	Ile	Pro	Ser	Ala	Ile	Ala	Ala	Asn
				105					110					115					120					125
UCC·	GGC·	AUC·	UAC·	UAA·	UAG·	ACG·	CCG·	GCC·	AUU·	CAA·	ACA·	UGA·	GGA·	UUA·	CCC·	AUG·	UCG·	AAG·	ACA·	ACA·	AAG·	AAG·	(U)	
Ser	Gly	Ile	Tyr														Ser	Lys	Thr	Thr	Lys	Lys		
			129														1				5			

Fig. 48. Nucleotide sequence of the coat protein gene of coliphage MS2 together with the amino acid sequences specified by this gene. The gene is preceded and followed by nongenic regions; note the initiating codon (AUG) preceding the first codon of the coat protein and the double terminating codons (UAA and UAG) at the end of the gene. The numbers refer to the positions of amino acid residues in the coat protein (1–129) and the first six in the replicase gene. (From Min Jou et al. 1972.)

Table 46. Codons Used in Phage MS2 Coat Protein Gene.[a]

Ala	GCA (6)[b]	Gly	GGA (2)	Pro	CCA (2)
	GCC (2)		GGC (3)		CCC (1)
	GCG (1)		GGG (1)		CCG (1)
	GCU (5)		GGU (3)		CCU (2)
Arg	AGA	His	CAC	Ser	AGC (4)
	AGG		CAU		AGU
	CGA				UCA (2)
	CGC (1)	Ile	AUA		UCC (2)
	CGG		AUC (4)		UCG (2)
	CGU (3)		AUU (4)		UCU (3)
Asp	GAC (3)	Leu	CUA (2)	Thr	ACA
	GAU (1)		CUC (2)		ACC (4)
			CUG		ACG (1)
Asn	AAC (6)		CUU (2)		ACU (4)
	AAU (4)		UUA (1)		
			UUG	Trp	UGG (2)
Cys	UGC (1)				
	UGU (1)	Lys	AAA (5)	Tyr	UAC (4)
			AAG (1)		UAU
Glu	GAA (2)				
	GAG (3)	Met	AUG (2)	Val	GUA (3)
					GUC (4)
Gln	CAA (1)	Phe	UUC (1)		GUG (3)
	CAG (5)		UUU (3)		GUU (4)

[a]Adapted from Min Jou et al. 1972.

[b]Numbers in parentheses refer to number of times codon is used in the gene.

It should be pointed out here that, although no examples have been given to illustrate the production and consequences of mutations of animal viruses, there has been extensive activity in the area of animal virus genetics involving a wide variety of animal viruses and numerous mutagenic agents. A comprehensive review of this subject is that of Ghendon (1972).

3. *Gene Location*

All viruses contain in their nucleic acids linear sequences of nucleotides that function through the processes of transcription and/or translation to yield specific proteins. These protein-generating segments of nucleic acid are called genes (or, by some, cistrons; see Benzer 1957). As indicated earlier the number of genes to be expected is proportional to the size of the nucleic acid, but all viruses are polygenic, meaning that they have enough nucleic acid to code for several proteins. The simplest RNA phages, such as MS2, R17, f2, and others like them, have only three genes,

as described in the previous section, whereas the large T phages and the pox viruses have enough nucleic acid to provide for 200 to 300 genes.

It has been shown for MS2 and R17 phages that the viral RNA, which serves both as genome and mRNA, does not consist entirely of genes. Some untranslated portions of the RNA serve other functions such as ribosome binding sites (Steitz 1969). There are doubtless similar untranscribed segments of double-stranded nucleic acids; in addition it appears that only one of the two strands of DNA is transcribed into messenger RNAs and hence translated into proteins. T4 and lambda coliphages are partial exceptions, one strand of the DNA being transcribed for certain genes and the other strand for the remaining ones (Guha and Szybalski 1968; Cohen and Hurwitz 1968).

A crude but rapid estimate of the number of genes in a viral nucleic acid may be obtained by the relation:

$$\text{Number of genes in single-stranded nucleic acid} = \frac{\text{Molecular weight of nucleic acid}}{0.3 \times 10^6}$$

For viruses with double-stranded nucleic acid, the molecular weight of the nucleic acid is divided by 2 in order to reflect the assumption that gene function is associated with only one strand. The assumptions behind this very approximate calculation are that most of the coding strand of nucleic acid is involved in gene function, that the average molecular weight of a nucleotide is 300 and of an amino acid 100, that the proteins coded for have a molecular weight of 35,000, and that the code is a triplet nonoverlapping one.

The sizes of viral genes vary considerably in accordance with the gene concerned. Thus the gene for the coat protein of phage fd has only 147 nucleotides (Asbeck et al. 1969), whereas genes for some of the larger viral proteins must consist of more than a thousand nucleotides.

Whatever their size or number, several methods have been used to determine the location of genes in viral nucleic acids: (1) mating and mapping; (2) hybridization and electron microscopy; (3) selective mutagenesis; and (4) comparison of amino acid and nucleotide sequences.

a. Mating and Mapping

The mating and mapping procedure has been used to locate genes in phage nucleic acids (Hayes 1968). The procedure depends on genetic recombination between strains of a virus when they infect the same cells, and mapping is based on the probability principle that the number of recombinants obtained will be proportional to the distance separating the genes that are recombined. Consequently, if virus strains are available that have distinctive hereditary characters that can be used as markers, crosses

Table 47. Recombination from Mating of Some Coliphages.

Phages Mated	Progeny				% Recombinants[a]
	Number and Types of Plaques				
Rc × Rm	5162 Rc (large, clear)	6,510 (minute, turbid)	311 RR (large, turbid)	341 cm (minute, clear)	5.3
Rs × Rc	7,101 Rs (small, turbid)	5,851 Rc (large, clear)	145 RR (large, turbid)	169 cs (small, clear)	2.4
Rs × Rm	647 Rs (small, turbid)	502 Rm (minute, turbid)	65 RR (large, turbid)	56 sm (small turbid with halo)	9.5

[a]Adapted from Kaiser (1955). Mutants designated here as Rc, Rs, and Rm were obtained by ultraviolet irradiation of a reference type of phage lambda designated RR. Percent recombinants $= \frac{\text{Sum of two recombinants}}{\text{Sum of all types}} \times 100$.

(mating) can be made between pairs of strains. From the results of the mating, the genes associated with the marker characteristics can be mapped. In a typical cross between two strains, each of which has one distinctive hereditary characteristic, four types of progeny are observed: two are the two parental types and two are new types that combine characteristics of the parental types (these are the recombinants). The results obtained in successive matings of pairs of plaque type mutants of a lambda phage variant are indicated in Table 47. From these data a linkage map can be constructed showing the relative positions of three genes that affect plaque type, as follows. Let each percent recombinants be equal to one map unit. Then the c and m genes are separated by 5.3 map units, while the c and s genes are 2.4 map units apart. This information is not enough to decide between c--s--m and s--c-----m, but after making the cross Rs × Rm, which indicated that s and m were separated by 9.5 map units, it is clear that the correct order of the genes must be s--c-----m. This is a simplified version of the simplest application of mating-mapping. In practice, allowances must often be made for such technical factors as differences in attachment efficiencies, repeated rounds of matings, and so on. Moreover, mating is often coupled with complementation tests in order to distinguish between mutants of the same gene and of different genes concerned with the same function; also, three-factor crosses, and deletion-mapping techniques are employed (see Hayes 1968).

b. Hybridization and Electron Microscopy

Another technique of gene location in DNA is by hybridization and electron microscopy (Davis and Davidson 1968; Westmoreland et al. 1969; Davis et al. 1971). The principle of this method is that the complementary strands of DNA can be separated (this is denaturation) by heat or other treatments and under optimal conditions brought back together (annealed) to form a structure that appears uniformly fibrous when viewed in the electron microscope. However, if two different DNAs are denatured and complementary strands from each are subjected to annealing, the double-stranded structure obtained (hybrid duplex) will show more or less uniformity depending on the degree of homology between the annealed strands. Nonpairing segments of nucleic acid form single-stranded loops that are seen as such in the electron microscope, or under some conditions the single-stranded loops collapse into small branched clumps called "bushes." If formamide is used in mounting DNA molecules for electron microscopy (Westmoreland et al. 1969; Davis et al. 1971), single-stranded regions of DNA do not collapse but appear in loops as filaments with measurable contour length. This latter technique, coupled with other labeling methods, has enabled the location of genes, such as those for tRNAs, in the DNA of coliphages (Wu and Davidson 1973); the formamide technique was used also to locate a bit of bacterial DNA incorporated into the DNA of

the transducing phage Ø80. This is illustrated in Figure 49, which shows the heteroduplex formed between a strand of Ø80 DNA and a complementary strand in which a segment of bacterial DNA containing genes for two tyrosine tRNAs has been substituted for a segment of phage DNA. The loopout section in Figure 49 represents the area of nonhomology between strands of DNA and permits the precise location of the tyrosine tRNA genes.

Morrow and Berg (1972) also used the hybridization-electron microscopy technique to demonstrate the presence and location of a small segment of simian virus 40 (SV40) genome in the hybrid species containing both adenovirus and SV40 DNAs. First, annealing of adenovirus-SV40 hybrid DNA with just adenovirus DNA produced a loopout of the SV40 segment, thus indicating its position in the hybrid DNA. Next, the specific

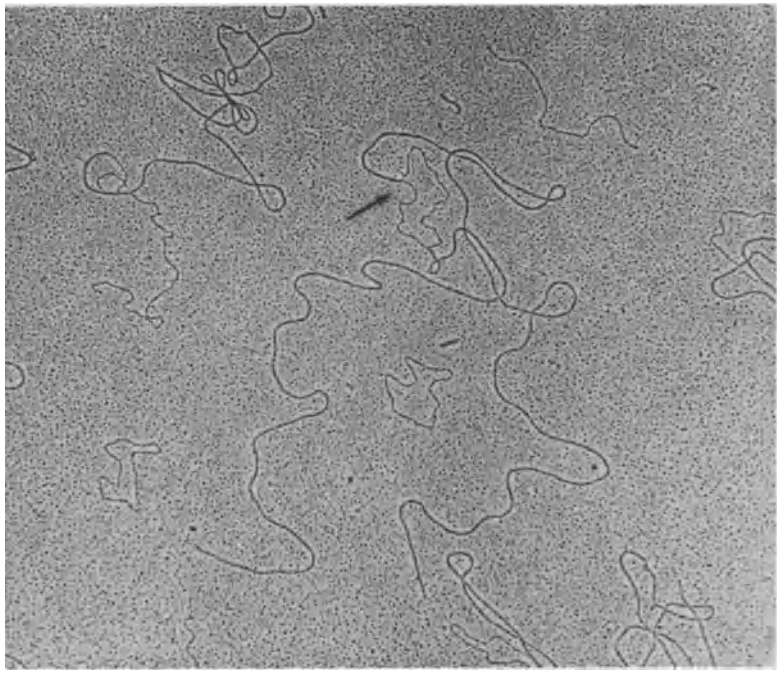


Fig. 49. Heteroduplex formed between a strand of coliphage Ø80 DNA and a complementary strand of Ø80 DNA in which a segment of bacterial DNA has been substituted for a segment of phage DNA; since there is no homology between these segments, they appear as single-stranded components of a loop in the otherwise uniform duplex. The arrow points to the junction of phage DNA and bacterial DNA, the longer part of the loop being bacterial DNA (about 3,100 nucleotides) and the shorter part, Ø80 DNA (about 2,100 nucleotides). The entire heteroduplex DNA molecule is shown; two single-stranded circular DNA molecules of phage ØX174 put in as length standards can also be seen. (From Wu and Davidson 1973.)

segment of SV40 DNA incorporated in the hybrid was identified by hybridizing the loopout heteroduplex with SV40 DNA. Hybridization produced a bit of triple heteroduplex by base-pairing between the loopout section and the complementary region on the added SV40 DNA. By inspection of the electron micrographs of this structure and by measurement of distances, it was possible to identify the specific region in the SV40 DNA from which the segment had come that was present in the adenovirus-SV40 hybrid. From other tests it was known that genes associated the SV40 DNA segment include one for a specific SV40 antigen and another enabling the hybrid to multiply in monkey kidney cells (adenovirus alone cannot multiply in these cells).

c. Selective Mutagenesis

There is little evidence that genetic recombination occurs with plant viruses, except for tomato spotted wilt virus (Best 1968). However, even with this virus, it is not yet possible to obtain the quantitative data on recombinants needed in order to locate genes by the mating and mapping procedures. Nor has the hybridization-electron microscopy method proved applicable to plant viruses thus far. However, a highly specialized procedure of selective mutagenesis, so far used only for tobacco mosaic virus, has been devised. The rationale of this procedure is based on the observation that the protein subunits spiralling around the tubular TMV particle can be stripped off from the RNA in a polar fashion starting from the end of the particle in which the 3′-terminal of the viral RNA is located (May and Knight 1965). This stripping is achieved by treating the virus with sodium dodecyl sulfate at 37°C for about 3 hr.

Large amounts of variously stripped particles obtained in this manner are separated into classes of different lengths by sedimentation of the reaction mixture on a sucrose density gradient. Such classes of particles are then treated with nitrous acid and tested for the production of a specific type of mutant (the nitrous acid can react mutagenically with viral RNA that is still ensheathed with protein but only at a fraction of the rate shown by the exposed RNA). A gene concerned with the viral capacity to produce brown necrotic spots on *Nicotiana sylvestris* tobacco in which the wild type causes a systemic green mottling response was located by this technique (Kado and Knight 1966). When the RNA segment containing the sought-for gene was exposed, the number of mutants increased significantly over background spontaneous mutants as shown in Figure 50.

d. Comparison of Amino Acid and Nucleotide Sequences

The most direct means of gene location is the sequencing of nucleic acid and identification of unique sequences with the amino acid sequences of specific proteins. This has been done with the MS2 phage as described in the previous section (Min Jou et al. 1972).

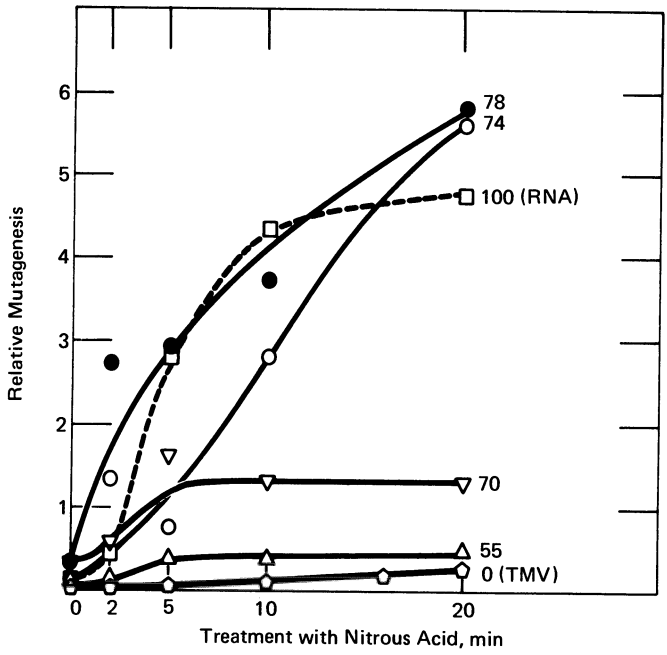


Fig. 50. Nitrous acid-induced mutagenesis of tobacco mosaic virus as a function of the fraction of the protein subunits that have been stripped from the viral RNA. Relative mutagenesis is the number of lesions on *Nicotiana sylvestris* tobacco (a quantitative measure of mutants only) per 1,000 lesions on *N. tabacum* L. var. *Xanthi nc* (a quantitative measure of total virus present). The figures at the ends of the curves refer to percent of stripping of protein from TMV particles prior to treatment with nitrous acid. (From Kado and Knight 1966.)

A somewhat less direct but related procedure was used with phage R17 RNA, which was employed as mRNA in cell-free synthesis of identifiable proteins (Jeppesen et al. 1970). As previously noted, there are three genes in the RNAs of the R17 series of phages that code for A protein (maturation protein), the major coat protein, and an RNA synthetase. Either whole phage RNA or parts of it can direct synthesis of these phage proteins *in vitro*. Two large segments of phage RNA were obtained by treatment with *E. coli* ribonuclease IV. One part, comprising 40 percent of the RNA included the 5′-end of the nucleic acid, while the remaining 60 percent contained the 3′-end. In a cell-free protein-synthesizing system with *E. coli* ribosomes it was found that the 60 percent fragment (3′-end) was translated to yield synthetase protein, while the 40 percent segment was not translated. However, when ribosomes from *Bacillus stearothermophilus* were used in the synthesis, only A protein was produced and this by the 40 percent fragment (5′-end). Thus it appears that the A protein gene is at the 5′-end and the synthetase gene is at the 3′-end; by difference, the gene for

the major coat is in the middle of the viral RNA. This was confirmed by some other experiments involving sequencing of small segments of phage RNA representing initiation regions and fragments of adjacent genes. It was possible to relate these gene fragments to one or another of the three phage proteins.

VI.

Reproduction of Viruses and Viral Constituents

In order to continue to exist, viruses must multiply. This they can do only in living cells, which provide the synthetic and energy-yielding mechanisms that viruses lack. The main features of virus reproduction (also called infectious process) are all known, although reams of information are still being published on detailed and esoteric aspects of the subject. There is only one major exception to this conclusion, the viroids, whose nature and mode of interaction with cells have yet to be clarified. Viroids will be treated in Sec. 1A.

A. Virus Reproduction in Cells

No form of life seems surely exempt from virus infection. Therefore a study of the infectious process naturally focuses on the interaction of a particular virus with some kind of cell—algal, bacterial, fungal, plant, vertebrate, or invertebrate. Presumably the ability of a given virus to infect a particular type of cell (specificity) has resulted from evolutionary events involving both cell and virus. Stages at which specificity might be exerted will be apparent as the infectious process is considered.

It is convenient to view the process of infection in terms of a sequence of events: attachment, penetration, multiplication, assembly, and release.

1. Simple Infection

Simple infection ensues when susceptible cells are infected by one or a few virus particles of the same kind. In complex or mixed infection, which will be treated in the next section, the consequences of simultaneous infection with active and inactive virus particles, with two or more mutants of a virus or with two or more unrelated viruses, will be briefly considered.

The systematic investigation of various steps in virus infection has advanced mainly where it is possible to study synchronous infection of cultures of bacterial, animal, and plant cells. In such systems, some complications of whole organisms can be avoided and quantitation of events is

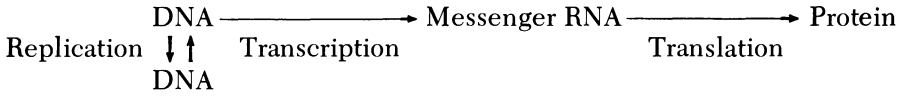
greatly enhanced. Cell cultures also lend themselves to application of radioisotopes, which are invaluable as markers of the various chemical constituents of viruses or of enzymes and other substances involved in virus multiplication.

The process of infection can be generalized for all viruses as follows.

Virus attaches to a susceptible cell at one of several specific sites where there is a structural and electrostatic complementarity between cell surface and virus particle. Some viruses appear to have attachment organs and some not (possible attachment organs include tail fibers or knobs of certain phages, penton fibers of adenoviruses, spikes of enveloped viruses, and so on). Specific attachment of a single virus particle is sufficient to initiate an infection; conversely, inability of a virus to attach specifically to the cell surface may block infection.

Following attachment, either whole virus or viral nucleic acid penetrates the interior of the cell. Penetration of bacterial and plant cells is potentially more difficult than invasion of animal cells since they have a cell wall as well as a plasma membrane to breach. Commonly it is whole virus that penetrates in infection by animal or plant viruses. Some animal viruses such as poliovirus can pass directly through the animal cell plasma membrane even when smaller particles are excluded; the molecular mechanism for this is as yet unknown. More commonly, virus particles are engulfed by a phagocytic type of reaction sometimes called viropexis. Quite often a virus may be partly degraded by cellular enzymes in the process of penetration either at the cell membrane or within phagocytic vesicles. Bacterial viruses and some algal viruses usually inject their nucleic acid into cells to which they have attached in a specific manner. The injection process is probably facilitated by such structures as contractile tail sheaths that some phages and algal viruses have. However, nucleic acid is injected by many bacterial viruses that have no such organ. The mechanism for this remains to be clarified.

Multiplication of viruses takes place in either the cytoplasm or in the nucleus or in both. In multiplication, the virus provides most essentially its nucleic acid which has the genetic information needed in order to produce more virus particles just like the one that initiated the infection. In addition, some viruses may bring in one or more enzymes needed in some step of the replication of viral nucleic acid. Beyond that, the cell provides energy-generating systems, enzymes, and synthetic machinery (especially protein-synthesizing equipment such as ribosomes, transfer RNAs, and so on) and raw materials from which virus constituents are made (amino acids for proteins, nucleotides for nucleic acid, and so on). Viral nucleic acid functions in virus multiplication in much the same way that it does in normal cellular function as prescribed by the central dogma of molecular biology:



In the case of RNA viruses in which the virion-contained RNA can act directly as mRNA, the production of virus-specific proteins may occur in two different ways: (1) translation of viral RNA may occur discontinuously in a manner that produces a series of discrete proteins, one for each gene (for example, R17 coliphage); (2) translation may produce a large polypeptide chain that is then cleaved enzymatically ("processed") into individual proteins with various functions (for example, poliovirus). If the viral nucleic acid is single-stranded RNA, it may function as a template for its own replication, as well as serve as messenger RNA (mRNA) that is translated on the cellular ribosomes to provide virus-specific enzymes and structural proteins. Some single-stranded RNAs have the wrong "polarity" and cannot serve directly as mRNA but must first be transcribed into a complementary strand. Likewise, double-stranded RNA, which some viruses have, substitutes for DNA in the scheme outlined above. In still another instance (for example, RNA tumor viruses) single-stranded RNA may be transcribed into DNA by a viral enzyme ("reverse transcriptase") and then the sequence shown in the central dogma scheme starting with DNA can apply. In any case, viral nucleic acid and protein are produced in abundance and may accumulate in pools until a critical concentration occurs, after which assembly of progeny virus particles begins. In other cases assembly may begin before measurable pools of virus parts accumulate. The process of virus multiplication is subject to controls at either the transcriptional or translational level. Many details of these controls are still to be elucidated.

Assembly of new virus particles can be largely or wholly a spontaneous process since such assembly has been observed with a few viruses in the test tube. However, in some cases, assembly may require specific enzymes to catalyze certain steps of the process. As has already been noted, some viruses mature at cell membranes of one sort or another where they appear to acquire both viral and host materials.

Mature virus particles are finally released from infected cells ready to begin a new cycle of infection. Release can occur in one or more of three ways: (1) the infected cell may lyse releasing virus particles and cellular constituents into the surrounding area; (2) the virus may pass out through tubular structures that develop in the cell (or which connect plant cells and are there called plasmodesmata); or (3) the virus particles may mature at the plasma membrane from which they bud in a process that is the reverse in some respects of the phagocytic entrance mechanism. The yields of virus obtained from infected cells vary considerably with virus and host but range from less than 100 per cell to more than 1,000 for bacterial viruses, and usually run around 10^5–10^6 particles per cell for animal and plant viruses.

There are known instances in which the multiplication of bacterial or animal viruses is postponed after initiation of infection. The deferred production of bacterial viruses is called lysogeny; a more general term that includes animal viruses (or others if the phenomenon is eventually observed with them) is virogeny. The essence of virogeny is that the viral nucleic acid appears to be integrated into host nucleic acid where it multiplies only as the cellular DNA replicates and where its genes are usually considerably restricted in activity. In the case of lysogenized bacteria, for instance, only the gene or genes concerned with production of a repressor for the other viral genes is active. In virogenic conditions involving some DNA tumor viruses of animals, some viral genes that code for certain viral antigens remain active while viral structural genes cease functioning. The mechanism of repression of these latter genes is still obscure.

Virogenic cells can be changed into a productive state ("induced") by radiation, especially ultraviolet, or by treatment with certain chemicals, such as nitrogen mustards, mitomycin C, iodouracildeoxyriboside (IUDR) and others. Also, if the integrated genome is brought into a different environment, induction may occur and normal production of virus ensue. This happens with lysogenic bacteria if the bacterial chromosome containing integrated virus genome is transferred from one bacterial cell to another in the process of conjugation. This is called zygotic induction. Likewise, if a nonpermissive cell (a cell which may integrate viral nucleic acid but not support production of whole virus) containing integrated SV40 is fused with a permissive cell, infectious virus is produced by the fused cell (called a heterokaryon).

When viral DNA is released from integration with cellular DNA, the release may be clean, or part of the viral genome may be left behind and a piece of the cell genome brought out in a virus particle. If the piece of cellular DNA thus acquired contains a gene for a function (for example, an enzyme) lacking in another cell, the latter cell may serve to detect this, for after infection it will show a functional capacity it previously lacked. This process is called transduction. When specific genes only are transduced, the process is called specialized transduction, and when a variety of genes is transduced, the extreme being when the phage particle contains no phage DNA but only bacterial DNA, the phenomenon is termed general transduction.

2. *Complex or Mixed Infection*

Interesting molecular genetic phenomena occur when, instead of infection by a single type of active virus particle, mixed infection occurs with virus mutants, unrelated viruses, or various mixtures of active and inactive virus particles. Mating, marker rescue, and multiplicity reactivation are three examples of phenomena occurring during mixed infection, all of

which appear to depend on the same process, that is, genetic recombination.

Mating has been reported for bacterial, animal, and plant viruses. The process is essentially the same for all of them. Mixed infection is made with, for example, two mutants of a virus that possess distinctive characteristics (genetic markers). The observed consequence of mixed infection is that four types of viral progeny issue: two of them are exactly the same as the two parental types, but the other two have combinations of characteristics of each of the parental types. This might be illustrated with two mutants of phage T2 whose plaque types are indicated by r and r^+ and whose host ranges are specified by h^+ and h:

$$T2rh^+ \times T2r^+h \rightarrow T2rh^+ + T2r^+h + T2rh + T2r^+h^+$$

In marker rescue mixed infection with a fully active mutant and an inactivated mutant can result in appearance ("rescue") of a genetic trait from the inactivated virus in an active virus. For example, Kilbourne and associates (1967) infected chick embryos with active A_2 influenza virus and heat-inactivated A_0 influenza virus. They obtained among the progeny of this mixed infection a viral strain having the hemagglutinin and internal (NP) antigen of A_0 parent but the neuraminidase of the A_2 strain. Thus the internal antigen and the hemagglutinin of the A_0 strain were rescued.

Multiplicity reactivation is operative when a single type of virus greatly inactivated, for instance, by ultraviolet light shows little infectivity when tested at a low multiplicity of infection (few virus particles per cell) but a disproportionately high infectivity when tested at a high multiplicity of infection.

All three of these phenomena are thought to be explained by genetic recombination between the participating molecular species. While details of the process are yet unclear (see Clark 1971; Davern 1971; Radding 1974), genetic recombination appears to involve breakage and reunion of nucleic acid molecules in such a way that there is a reciprocal exchange of parts of the nucleic acids. This is illustrated graphically in Figure 51 for the mating between phages outlined above; by changing designations of the markers the scheme will apply as well to marker rescue and multiplicity reactivation.

A special type of genetic recombination that does not require the breaking-and-reunion recombinational mechanism cited above has been observed with influenza virus and could probably occur with any virus having a segmented genome. Since the nucleic acid of influenza virus occurs in several segments, the mixed infection of a cell with two mutants of this virus can presumably lead to the production of progeny segments that can be randomly assorted at the time of assembly into virus particles. This process is perhaps properly called genotypic mixing, and is thought to be responsible for the high rate of apparent recombination observed with influenza viruses.

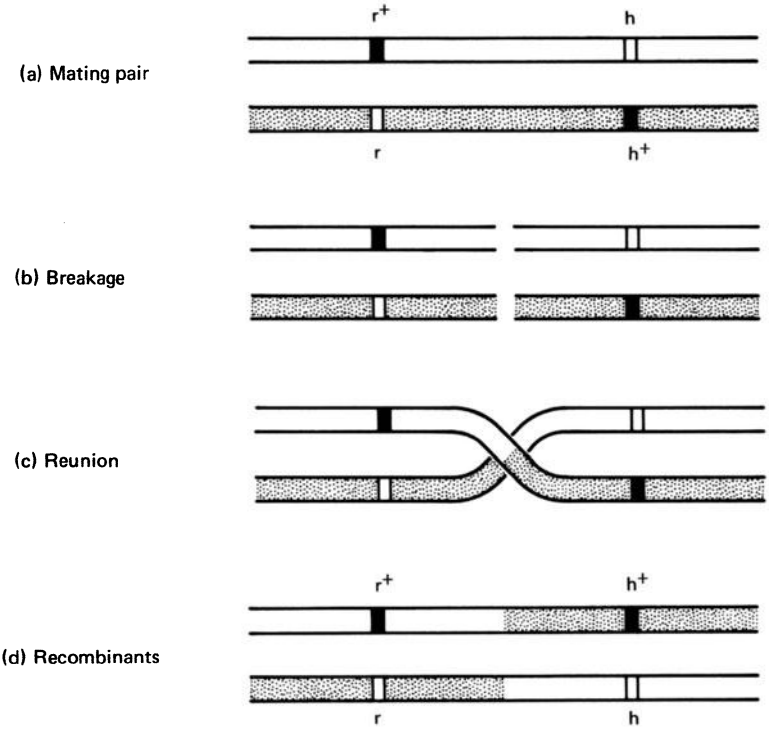


Fig. 51. Diagrammatic representation of genetic recombination between two phages by a mechanism involving breakage and reunion of their DNAs.

Some other phenomena of mixed infection that, however, do not involve genetic recombination include phenotypic mixing, complementation, and interference.

When the infectious process involves accumulation of viral parts in the metabolic pool before assembly begins, it is particularly feasible for some particles to be assembled from components of different viruses. This is phenotypic mixing. For example, mixed infection of *E. coli* bacteria with phages T2 and T4 might result in the production of some particles having all the usual constituents of T2 but the tail fibers of T4. Such a particle, upon infecting a fresh *E. coli* cell, will yield only T2 progeny since the DNA of the mixed particle is that of T2 phage.

When two viruses having different defects or deficiencies infect the same cell, they can sometimes supply each other with the missing function and thus make it possible for each to multiply whereas alone they could not. This is called complementation and has been observed many times with different viruses. Complementation may also involve defective (satellites) and nondefective viruses (Kassanis 1968; Barrett et al. 1973). A kind

of complementation that seems to be very common among certain plant viruses is that which occurs between functionally different particles (Sänger 1968; Lister 1969). An example of this is tobacco rattle virus, preparations of which characteristically display short and long rods (see Figure 35). The RNA of the short rods apparently codes for coat protein for both short and long particles, while the RNA of the long rods codes for the RNA polymerase needed for both types of particles. Thus the short rods are noninfectious by themselves; the long rods are infectious but induce the production of viral RNA only.

Some mixed infections result in competition and interference between different viruses presented to a cell. The mechanisms for interference are not always clear but as a rule dissimilar phages tend to interfere mutually in the infectious process; conversely, similar plant viruses (strains or variants) tend to interfere with each other. In some instances, it appears that the interfering viruses may compete for attachment sites; in other cases one virus may induce the production of a nuclease enzyme that destroys the nucleic acid of the second virus; still another possibility is that the nucleic acid of one virus may compete better for cellular ribosomes than the other, and so forth.

Detailed accounts of the infectious process, either simple or mixed, for a variety of viruses can be found in various works. See, for example, Knight 1974; Fenner et al. 1974; Dalton and Haguenau 1973; Matthews 1970; Mathews 1971; Hayes 1968.

3. *Viroids*

Several infectious diseases of plants closely resemble virus infections except in one important regard: no virus particles can be isolated from the diseased plants. The best-studied cases are potato spindle tuber disease and citrus exocortis, although chrysanthemum stunt is also an agent of this type. In all cases the agent appears to be a single-stranded RNA about 75,000–100,000 in molecular weight (Diener 1972; 1973; Semancik et al. 1973; Diener and Smith 1973.) Diener proposed the term viroid for such agents, thus indicating their viruslike properties but suggesting basic differences.

Viroids pose two puzzling major questions: (1) How does such a small nucleic acid get replicated? (2) How does such a small nucleic acid exert its pathological effects?

A standard concept, definitely supported by evidence in some cases, of how virus nucleic acids get duplicated in cells they infect is that they code for a polymerase (replicase) enzyme that can use the invading nucleic acid as a template for synthesis of more viral nucleic acid. If all the viroid RNA is used as mRNA, a replicase of molecular weight of only about 8,000–10,000 could be made. This seems much too small in comparison with

known plant RNA polymerases (Astier-Manifacier and Cornuet 1971; Peden et al. 1972; Hariharasubramanian et al. 1973) even if such a protein serves to modify an existing replicase. It is possible that the viroid RNA is replicated by a host enzyme but no such enzyme has yet been identified. If the viroid RNA acts as mRNA for a nonreplicase protein, could that protein account for the pathologic activity of viroids? The answer is unknown. Perhaps the viroid RNA causes pathologic effects by exerting some kind of abnormal regulatory control of cell processes, perhaps in the nucleus. If so this action has yet to be defined. Thus viroids represent mysterious virus-like entities whose structure, mode of replication, and pathogenic mechanism remain unknown.

B. Extracellular Reproduction of Viruses and Viral Constituents

Viruses are obligate parasites and as such do not replicate independently outside living cells. However, in the laboratory it has been possible to obtain virus constituents and to put them together in such a way as to reproduce the morphology and biological activity of certain viruses. This is called reconstitution. It has also been possible to induce cell-free synthesis of some viral proteins and nucleic acids in the laboratory.

1. Reconstitution

Reconstitution (sometimes referred to as self-assembly) is defined as the bringing together of protein and nucleic acid components of a virus in such a way that they combine to yield characteristic virus particles possessing most, if not all, of the properties (including, in the most successful cases, infectivity) of the mature virus as it is produced in natural infections. The component parts for reconstitution are obtained by degrading the virus, although sometimes the protein component is available as a natural consequence of infection and can be isolated and used in the reconstitution reaction. Reconstitution was first accomplished with TMV and some of its strains by Fraenkel-Conrat and Williams (1955) building on the results of experiments of Schramm (1947a, 1947b) and especially those of Takahashi and Ishii (1952a, 1952b, 1953). Takahashi and Ishii had demonstrated that the TMV protein found in infected plants could be aggregated to form rods that in the electron microscope looked very much like TMV but which were devoid of infectivity since they lacked RNA.

While there are several ways of obtaining protein and nucleic acid for demonstrating and studying the reconstitution of TMV, the RNA is now commonly obtained by the phenol extraction method (see Sec. IIIB, 1) and the protein by the cold 67 percent acetic acid procedure (see Sec. IIIA, 1).

Protein prepared by the latter procedure occurs at around neutral pH in the form of quasistable aggregates composed of three subunits and generally called A protein. The reconstitution reaction can be summarized for TMV and its strains as follows:

A protein	+	RNA	$\xrightarrow[\text{pH 7.3}]{\text{1 hr at 30° C}}$	Reconstituted virus
			Pyrophosphate	
Molecular Weight 53,000		Molecular Weight 2×10^6		Molecular Weight 40×10^6
Noninfectious		Slightly infectious		Highly infectious 18×300 nm rods

As indicated, the protein used in reconstitution has no infectivity and the infectivity of the RNA usually amounts to about 0.05 percent that of the same amount of RNA in a virus particle. In contrast, the reconstituted virus exhibits infectivities ranging from 30 to 100 percent of those in the virus from which parts were obtained for the reconstitution. The structure of reconstituted virus as revealed by electron microscopy (see Figure 52 for reconstitution sequence), the ultraviolet absorbance (Figure 53), and the stability shown to heat and various pH values by reconstituted virus are virtually identical to those exhibited by the virus obtained from infected tobacco plants.

Much is known about the details of the TMV reconstitution. The factors involved in association of the protein subunits, especially the role of water, have been exquisitely analyzed by Lauffer and Stevens (1968). They cite evidence to support the idea that the polymerization of TMV A protein is an endothermic aggregation reaction and that the observed increase in entropy is associated with the release of bound water between the polymerizing units. Thus polymerization of TMV A protein can be thought of as a transfer of organic surface from an aqueous environment to an organic environment and the importance of hydrophobic interactions in holding the superstructure of TMV rods together is stressed. In reconstitution of whole TMV there are, of course, interactions (also noncovalent) between protein and nucleic acid as well as between protein and protein (see Caspar 1963). These are important as evidenced by the fact that TMV protein rods readily disaggregate above pH 8, whereas rods containing the viral RNA are stable up to pH 10.

The composition, size, and charge of the viral protein influence the process of viral reconstitution, as can be demonstrated in three ways:

1. Mixed reconstitution can be done by using the RNA of one strain and the protein of another but the reconstitution may not go so well in this heterologous reaction especially when it involves strains with grossly different proteins (Holoubek 1962); another instance emphasizing the impor-

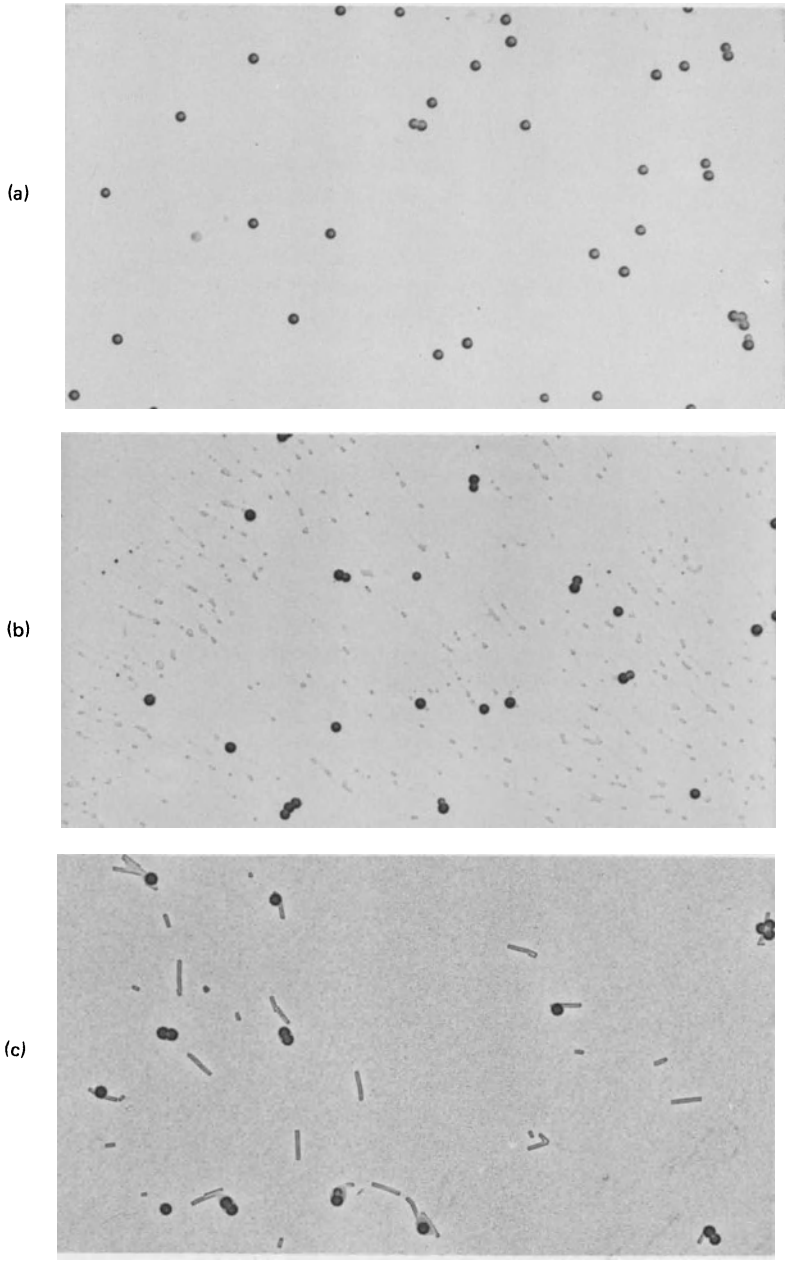


Fig. 52. Electron micrographs of a reconstitution mixture of tobacco mosaic virus A protein and tobacco mosaic virus RNA at various times after mixing. *a*. Immediately after mixing: only polystyrene latex reference spheres are visible; *b*. 2 min after mixing: many fibrous nucleic acid particles are visible, each of which has some protein assembled around one end; *c*. 6 hr after mixing: many full-size TMV particles are present. (Courtesy K. Richards.)

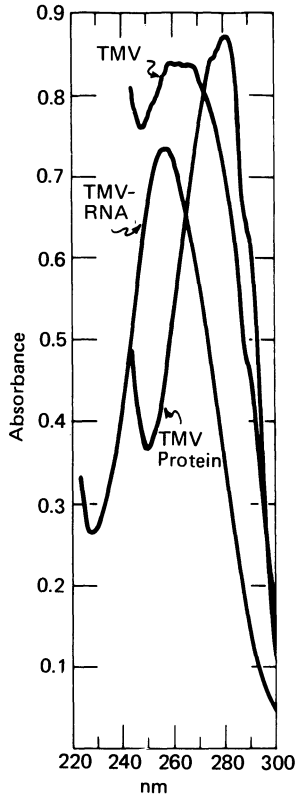


Fig. 53. Ultraviolet absorption spectra of tobacco mosaic virus, its coat protein, and its RNA.

tance of composition of the viral protein in reconstitution is the observation by Siegel and Zaitlin (1965) that the PM 2 strain of TMV obtained after nitrous acid mutagenesis of common TMV has a protein differing from that of common TMV by only two amino acid replacements but that this protein will not aggregate around viral RNA in the reconstitution reaction and aggregates by itself to form a bizarre open-helical structure.

2. If three C-terminal residues are removed from each protein subunit, reconstitution of rods is unimpaired but if 15 to 17 amino acid residues of the 158 per subunit are removed, the ability to form rodlike particles is removed (Sengbusch and Wittmann 1965).

3. The protein of common TMV has two lysine residues per subunit (at positions 53 and 68, see Table 13) and the epsilon amino groups of these residues are normally protonated; these charges can be selectively eliminated by trifluoroacetylation, which renders the protein incapable of aggregation to rodlike structures, but this capacity is restored by removal of the trifluoroacetyl groups (Perham and Richards 1968).

From the point of view of the mechanics of reconstitution of TMV, it appears from Richards and Williams' (1972) analysis that an aggregate of 34 protein subunits in the form of a disk (which arises spontaneously when A protein concentration and pH are suitable) is essential for rod initiation; addition of individual units of A protein then results in rod elongation. The process seems to be polar, the addition of protein proceeding from the 5′-end of the viral RNA toward the 3′-end (Dzhavakhia et al. 1970; Hirth 1971).

Several other viral RNAs as well as synthetic polynucleotides have been reconstituted with TMV protein in place of TMV-RNA (Fraenkel-Conrat 1970). Rodlike particles were generally obtained whose average length varied with the nucleic acid employed. Slight infectivity in Chinese cabbage was observed for the reconstituted virus containing turnip yellow mosaic virus RNA (Chinese cabbage is a common host for the turnip virus).

Another rodlike virus, tobacco rattle virus has also been successfully reconstituted, although the conditions favoring reconstitution are somewhat different from those used with TMV (Semancik and Reynolds 1969; Morris and Semancik 1973). Protein was extracted from the virus with acetic acid and RNA was obtained by a phenol extraction procedure. The two components were mixed in the proportions of 10 parts of protein to 1 of RNA and dialyzed against 0.25 M glycine buffer at pH 8 at 9°C for 12 hr. Nucleoprotein sedimenting at the same rate as native virus and resembling the latter in appearance in the electron microscope was obtained. This material exhibited infectivity.

The first demonstration of reconstitution of spheroidal viruses was not achieved until some years after success with TMV. This may be due to the apparently greater susceptibility to denaturation of proteins from spheroidal viruses; at least the earlier subjects for investigation such as tomato bushy stunt and turnip yellow mosaic viruses are in the category characterized by coat proteins that are readily and often irreversibly denatured.

However, Hiebert et al. (1968) (see also Bancroft 1970) found that spheroidal plant viruses of the brome mosaic group (for example, brome mosaic, cowpea chlorotic mottle, and broad bean mottle viruses) could be readily disaggregated and reconstituted. These viruses are serologically unrelated but share certain chemical and physical properties (particles are about 25 nm in diameter and contain about 22 percent RNA and 78 percent protein) and also have some common hosts. The RNA can be obtained from them by the phenol extraction procedure and the protein by dialyzing virus against a mixture containing 1 M NaCl, 0.02 M tris buffer (pH 7.4) and 10^{-3}M dithiothreitol (Cleland's reagent) at 4°C for 24 hr. The reaction mixture is centrifuged to separate virus protein from undegraded virus and RNA. Reconstitution is achieved by dialyzing 1 part of viral RNA and 3 parts of protein for 1 hr at 4°C against a tris-salts mixture (0.01 M tris at pH 7.4, 0.01 M KCl, 5×10^{-3} M $MgCl_2$, and 10^{-3} M dithiothreitol).

In addition to homologous reconstitution, hybrids can be formed that

have the protein coat of one virus and the RNA of another. All are infectious, and the hybrids show a host range specificity characteristic of the virus from which the RNA was obtained. It was also shown that the protein from cowpea chlorotic mottle virus could reconstitute with RNAs of certain other viruses and phages, and even with a phage DNA as well as with soybean ribosomal RNA and yeast sRNA. In all cases spheroidal particles were obtained with a size similar to that of the cowpea virus. Thus, as with the TMV series, the protein constituent tends to determine the size and shape of reconstituted particles, with the reservation that the length of stable rodlike particles may depend on the length of the RNA used in the reconstitution.

Other plant viruses have also been dissociated and reconstituted but with somewhat less certain recovery (or preservation) of infectivity (see Fraenkel-Conrat 1970).

Reconstitution has also been achieved with the spheroidal RNA phages of the f2 group (for example, f2, fr, MS2, R17) and with the similar Qβ phage (see Hohn and Hohn 1970). These phages are characterized by molecules of single-stranded RNA (molecular weight 1×10^6) in a protein shell whose major protein constituent is 180 subunits of protein, each about 13,750 in molecular weight. In addition, one molecule called A protein or maturation protein (molecular weight 35,000) must be present for an infectious particle. A difficulty in testing reconstitution of these viruses is that their proteins are difficultly soluble by themselves, the A protein being especially insoluble. However, if the RNA is obtained by phenol extraction and the protein by treatment with ice-cold dilute acetic acid or guanidine hydrochloride, these components can be mixed and dialyzed against renaturing buffers (usually tris-salt mixtures similar to those previously described) to yield particles similar to native virus in size and shape. If reconstitution is done in the absence of A protein, no infectivity is found with the reconstituted particles; but it has been possible to get particles with low but definite infectivity in the presence of A protein.

Among the animal viruses, it has been reported that poliovirus can be reconstituted if a rather special procedure is employed (Drzeniek and Bilello 1972). Poliovirus can be dissociated into its protein (four species) and nucleic acid (single-stranded RNA) components by holding it at 25°C for 60 min in 10 M urea and 0.1 M mercaptoethanol. Infectious poliovirus can be reconstituted from its parts by diluting the urea dissociation mixture in five steps in cold phosphate-buffered saline at pH 7.2. Attempts to reconstitute by a one-step dilution or by dialysis have been unsuccessful.

Some of the structurally complex, tailed coliphages have been partly reconstituted by mixing phage parts under appropriate conditions. This has been done with the coliphages T4 and lambda (Wood and Edgar 1967; Edgar and Lielausis 1968; Weigle 1966; Casjens 1971) and with the *Salmonella* phage P22 (Israel et al. 1967). Appropriate conditions for assembly

of these phages are simply incubation in dilute salt solutions at 30°–37°C for 1–2 hr.

For example, a magnesium-containing phosphate buffer at pH 7.4 of the following composition was used in some of the T4 reconstitutions: 0.0039 M Na_2HPO_4, 0.0022 M KH_2PO_4, 0.007 M NaCl, and 0.02 M $MgSO_4$. Advantage is taken here of conditional lethal mutants which, under restrictive conditions (such as elevated temperature), are unable to produce all of the phage parts required for infectious particles. By matching mutants whose parts are complementary, complete phage can be assembled, providing that the complementary parts will unite spontaneously. For example, a certain mutant of T4 when grown at an elevated temperature produces phage particles that are complete except for tail fibers. Since tail fibers are the attachment organs for T4, the particles lacking these are not infectious. Such defective particles can be isolated from artificial lysates (infected cells caused to burst by treatment with chloroform). Similarly, another mutant defective with respect to phage head formation produces many tail fibers that can be isolated from artificial lysates of the infected cells. These fibers, incubated with the tail fiberless particles, self-assemble to form complete, infectious particles. Since such assembly is often tested with extracts containing the complementary phage parts, the reconstitution procedure has been termed extract complementation. Aside from its use in studying phage assembly, extract complementation can sometimes be employed to determine whether the same or different morphogenetic genes have been affected in a series of mutants.

While it is clear that some portions of phage assembly can occur spontaneously, there is good evidence that some steps in the process with the structurally complex phages may require specific enzymes coded for by the virus.

A partial reconstitution of lambda phage involves the coupling of phage heads and tails. This assembly resembles the situation with the tailless RNA phages in the sense that a protein molecule (molecular weight 17,000) called the F product (that is, F gene product) is required. Thus a mixture of lambda heads and tails is no more infectious than either alone, but if F protein is included, the infectious titer rises several orders of magnitude (Casjens 1971). The lambda F protein, like the A protein of R17 phage, is thought to be essential in completing the phage head assembly. Lambda tails apparently do not join effectively to heads lacking F protein.

2. *Cell-free Synthesis of Viral Proteins*

In general, the proteins of viruses are so large that their synthesis in the laboratory by chemical techniques employed in peptide syntheses would be a major if not impossible undertaking. However, much has been learned about the basic mechanism of protein synthesis by studies with cell-free

systems (see Lucas-Lenard and Lipmann 1971), and such systems have been employed in the laboratory synthesis of small amounts of some viral proteins. One difficulty of such procedures is that all viral nucleic acids are polygenic (polycistronic) so that many mRNAs are made in an infected cell from a viral DNA, or if an RNA virus is used, the RNA also codes for several different proteins. This makes *in vitro* synthesis of a specific viral protein difficult. However, this problem is minimized with the small RNA phages, which have just three genes: A or maturation protein, main coat protein, and RNA synthetase or replicase.

Cell-free synthesis of viral proteins of such phages can be summarized as follows. The RNA is isolated from purified phage by the phenol extraction procedure. This serves as mRNA. Ribosomes are obtained from *E. coli* cells by extraction with dilute salt and centrifugal clarification. Such preparations of ribosomes usually contain tRNAs (although in some experiments these are prepared separately and added to the system) and various other factors needed such as those required for polypeptide chain initiation, elongation, termination, and so on. ATP and GTP are added, as well as an ATP-generating system such as phosphoenolpyruvate and pyruvate kinase. Amino acids are usually added, including one or more radiolabeled ones (amino acids are present in the ribosomal extract as well). Mg^{++}, KCl, and tris buffer (pH 7.4–7.8) are added to complete the mixture. The mixture is incubated at 25°–35°C for 30 min and then treated with trichloroacetic acid (TCA) which precipitates the protein that has been synthesized along with that in the ribosomal extract. After suitable washing, this precipitate is analyzed by one or more procedures to identify viral protein that may have been synthesized. Since this kind of synthesis is rather inefficient and hence involves only microgram amounts, cold (nonradioactive) viral proteins are sometimes added prior to analysis. Proteins from the reaction mixture can be separated on DEAE cellulose or Sephadex columns or, very commonly now, by electrophoresis on polyacrylamide gels. Further characterization of isolated protein can be done by digesting with trypsin and comparing the resulting peptides with those obtained from protein isolated from purified virus. Such comparisons are conveniently made by a peptide mapping procedure such as was illustrated in Figure 14.

Synthesis of one or more of the virus-specific proteins of the RNA phages MS2, f2, R17 has been demonstrated in cell-free systems such as that sketched above (Eggen et al. 1967; Nathans et al. 1962; Capecchi 1966). Likewise, the cell-free synthesis of T4 lysozyme and of the major head protein of T4 coliphage have been reported (Coolsma and Haselkorn 1969; Klagsbrun and Rich 1970).

Attempts to synthesize plant viral proteins in the *E. coli* system using tobacco necrosis satellite RNA and alfalfa mosaic virus RNA as messengers yielded proteins that gave tryptic peptide patterns similar to those of authentic viral proteins (Clark et al. 1965; van Ravenswaay-Claasen et al.

1967). TCA-precipitable material was also produced in the *E. coli* system when TMV-RNA was used as messenger, but the product could not be identified (Aach et al. 1964).

In contrast, both necrosis satellite RNA and TMV-RNA appear to be translated in cell-free extracts of wheat germ in such a way that resultant coat proteins can be identified (Klein et al. 1972; Roberts and Paterson 1973). Shih and Kaesberg (1973) made a particularly favorable application of the wheat germ system with RNAs isolated from the four different species of brome mosaic virus particles, one of which appears to be monogenic and codes for the viral coat protein. Looking at it in reverse, application of *in vitro* synthesis enabled the establishment of function of the brome mosaic RNA called RNA 4. In addition, it was found that coat protein exerted an inhibitory effect on *in vitro* translation of other viral RNA messages which may indicate a regulatory function in the *in vivo* protein synthesis induced by this virus. Such inhibition by coat protein had been noted earlier in an RNA phage system (Sugiyama and Nakada 1968).

The wheat embryo system has also been demonstrated to support *in vitro* protein synthesis by a phage ($Q\beta$) mRNA and an animal virus (vesicular stomatitis virus) mRNA (Davies and Kaesberg 1973; Morrison et al. 1974).

Several animal virus mRNAs have been translated in *in vitro* systems using cell-free extracts from a variety of cells (rabbit reticulocytes, Krebs II mouse ascites, Chinese hamster ovary, mouse L fibroblasts, HeLa, and so on). Some recent examples include vesicular stomatitis virus (Morrison et al. 1974), reovirus (McDowell et al. 1972), and Sendai virus (Kingsbury 1973). In all of these cases the objective has usually been to elucidate some feature of the viral replication process rather than to demonstrate cell-free synthesis of viral protein although the latter is a significant development of the past decade.

An important aspect of *in vitro* synthesis research with respect to the genetic code and mechanisms of protein synthesis is the demonstration that *E. coli*, rabbit reticulocyte, and wheat germ cell-free systems can translate messenger RNAs from various types of viruses. For example, the wheat germ system has been used with mRNAs from plant viruses, animal viruses, and phages. This emphasizes the universality of the genetic code and the similarity in fundamental mechanisms through which the code functions.

3. *Cell-free Synthesis of Viral Nucleic Acids*

Cell-free synthesis of viral RNAs or portions thereof have been accomplished for some years starting perhaps with the isolation of an RNA polymerase (also called synthetase, or replicase) from cells infected with

the RNA coliphage MS 2 (Haruna et al. 1963). Research soon shifted to a stabler enzyme produced in *E. coli* cells infected with Qβ phage. A review of these developments is given by Spiegelman et al. (1968), and a recent survey has been made by August et al. (1973).

A summary of the cell-free synthesis of phage viral RNA is as follows. The viral RNA is required as a template. This can be isolated from the purified virus by the phenol extraction procedure. The replicase enzyme is extracted from infected cells and used in a purified form obtained from extracts by density-gradient centrifugation and chromatography. To the template RNA and replicase enzyme in a tris buffer at about pH 7.4 containing magnesium ions are added four nucleoside-5′-triphosphates (ATP, GTP, CTP, and UTP), one or more of which is usually radioactively labeled. The mixture is incubated at about 30°C for 30–60 min. [The *in vitro* synthesis may require addition of one or more host factors (August et al. 1973), although if manganese ions are provided as well as Mg^{++} these may be unnecessary (Palmenberg and Kaesberg 1974).] The product of *in vitro* synthesis may be tested directly for biological activity or isolated as an acid-insoluble product by treating the reaction mixture with trichloroacetic acid. The quantity of product in the acid-washed precipitate can be estimated by radioactivity measurements.

Haruna and Spiegelman (1965) used such experimental conditions to effect the first *in vitro* synthesis of infectious nucleic acid. Since RNA phages have a penetration mechanism that their nucleic acid lacks, this infectivity was demonstrated by use of bacterial protoplasts called spheroplasts. These are bacterial cells whose walls have been removed by treatment with lysozyme.

The specificity of *in vitro* synthesis of Qβ RNA is usually quite high, occurring mainly with either plus or minus strands of Qβ RNA or RNA of its mutants as templates; however, by use of both Mn^{++} and Mg^{++} in the reaction mixture, some synthesis of coliphage R17 RNA and of brome mosaic and tobacco mosaic RNAs can be achieved (Palmenberg and Kaesberg 1974).

Several groups of RNA-containing animal viruses appear to incorporate virus-specific RNA polymerases in their virions. Such virions can then be used directly (or in some cases after treatment with nonionic detergents or proteolytic enzymes) in *in vitro* synthesis of viral RNA using conditions similar to those described above. The products observed in such syntheses thus far have been somewhat heterogeneous and variable in the completeness of synthesis; in contrast to the phage situation, no instance of production of infectious nucleic acid has been observed, but the animal virus RNAs are all much larger in size, which makes the task more difficult with respect to maintaining the integrity of template and product during the incubation period of the *in vitro* synthesis.

Some illustrative examples of the *in vitro* action of viral RNA-dependent RNA polymerases include those of myxoviruses, for example, influenza virus (Skehel 1971; Penhoet et al. 1971); diplornaviruses, such as reovirus; cytoplasmic polyhedrosis virus of the silkworm and wound tumor virus of sweetclover (Borsa and Graham 1968; Lewandowski et al. 1969; Black and Knight 1970); and rhabdoviruses, for example, vesicular stomatitis virus (Baltimore et al. 1970).

A stable, soluble poliovirus RNA-polymerase complex has been isolated from infected HeLa cells and shown to be active in the *in vitro* synthesis of poliovirus RNA (Ehrenfeld et al. 1970). The poliovirus polymerase complex differs from the examples above in that it is a subviral structure active in infected cells but not appearing in the mature virion, which is devoid of polymerase activity. Some plant virus RNA-dependent RNA polymerases have also been isolated in partially purified form from plant tissues infected with turnip yellow mosaic, cucumber mosaic, tobacco ringspot, and brome mosaic viruses but the *in vitro* synthesis products have yet to be more than superficially characterized (Astier-Manifacier and Cornuet 1971; May and Symons 1971; Peden et al. 1972; Hadidi and Fraenkel-Conrat 1973).

Many enzymes are known that are active in some phase of DNA synthesis, and valuable *in vitro* applications have been made of this knowledge.[1] However, few attempts at *in vitro* synthesis of viral DNAs have been made, probably because these DNAs are so large and the enzymes needed for the chore are scarce. Nevertheless, an outstanding *in vitro* synthesis of infectious DNA, paralleling the similar feat with RNA performed earlier by Haruna and Spiegelman (1965), was accomplished by Goulian, Kornberg, and Sinsheimer (1967). Infectious coliphage ØX174 DNA was produced in this *in vitro* synthesis summarized in Figure 54. The enzymes used were isolated from *E. coli*.

The virions of RNA tumor viruses have several enzymes associated with them, but the one most intensively studied so far is an RNA-dependent DNA polymerase called reverse transcriptase (Temin and Mizutani 1970; Baltimore 1970). For a while, this enzyme seemed to threaten the central dogma of molecular biology, that information flows from DNA to RNA to protein; reverse transcriptase was so named because information flow goes back from RNA to DNA and then subsequently forward in the usual direction. However, Crick, an originator of the central dogma, judged this anomaly to be within the basic concept (1970). In any case, *in vitro* synthesis of radioactive DNA segments using tumor virus RNA as template and viral reverse transcriptase provides valuable material with which to probe for tumor virus nucleic acids by hybridization reactions (see Tooze 1973).

[1]See *DNA Synthesis in Vitro*, R. D. Wells and R. B. Inman, eds., Baltimore: University Park Press (1973).

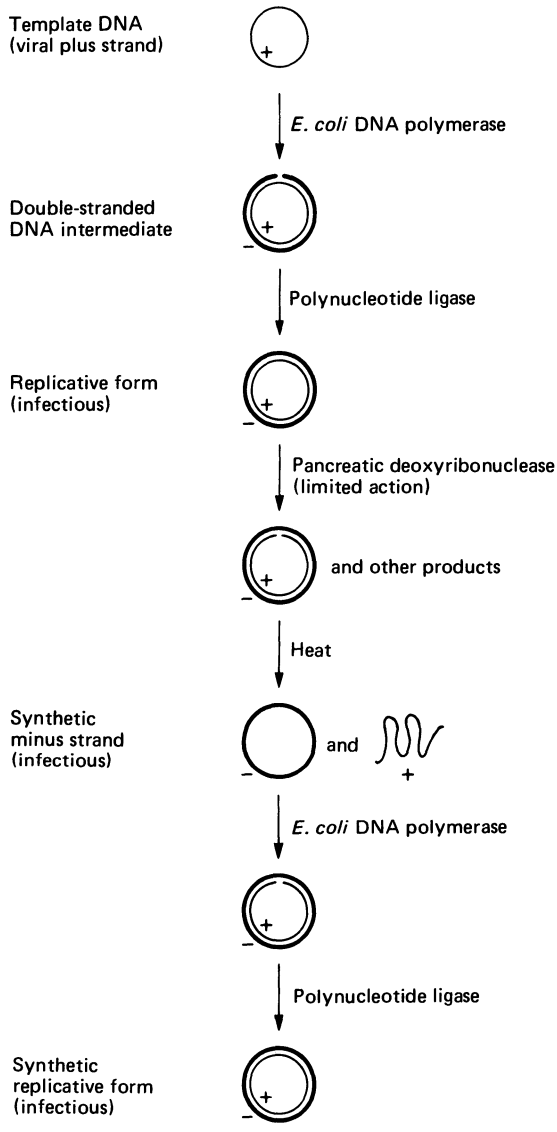


Fig. 54. Schematic representation of the enzymatic synthesis of infectious phage ØX174 DNA. (From Knight 1974 as adapted from Goulian et al. 1967 by omitting important but distracting details such as radioactive and density labeling and some of the products.)

C. Origin of Viruses

The origin of viruses and the broader question of the origin of life are matters for speculation. At least three ideas can be advanced for the origin of viruses: (1) viruses originated in early days of the planet by chemical evolution; (2) viruses arose from more complex microorganisms by a process of retrograde evolution; (3) viruses arose from the genetic material of cells.

Reconstruction experiments, starting with the raw materials and kinds of energy postulated to abound around primitive planets, have yielded amino acids, peptides, sugars, lipids, purine and pyrimidine bases, and many other organic compounds (see reviews by Fox et al. 1970; Fox 1971; Ponnamperuma and Gabel 1968). From the results of these experiments it is conceivable that nucleoproteins were among the products of chemical evolution. If it is imagined that such nucleoproteins had the ability to organize duplicate molecules from their surroundings, they might be thought of as primitive viruses. Furthermore, mutation of some of these primitive viruses may have led to more complex forms until the first cell-like assemblies were developed from which biological evolution may have proceeded. There is little to substantiate these conjectures at present.

Likewise, the notion that viruses may represent the products of retrograde evolution of higher organisms (for example, bacteria) has little to support it. There is an obvious progression in complexity from the simplest virus to the larger, more complex ones, from these to the simplest bacteria (for example, Bedsonia, Rickettsiae, and so on), and from there to the most complex bacteria and other protists. If this progression is reversed, one could imagine viruses as the ultimate product in morphologic and metabolic regression toward parasitism. However, such a series has not been induced experimentally and this idea remains speculative.

The most plausible concept of the origin of viruses seems to be that they represent detached segments of cellular DNA, or transcripts thereof in the case of RNA viruses, which achieved some measure of autonomy and evolved parallel with their hosts. Acquisition of protein coats and other morphologic structures exhibited by current complex viruses could be viewed as evidence of the operation of mutation and selection in the evolutionary process. Perhaps the most suggestive current model in support of the origin of viruses as escaped genetic elements is the bacterial plasmid. Plasmids are supernumerary DNAs of about 20×10^6 daltons that exhibit a variety of functions and accordingly are called sex factors, bacteriocinogenic factors, and resistance transfer factors. In a sense, they are all sex factors in that they share the property of promoting conjugation of and genetic transfer between bacterial cells. Those cells that have a sex factor transfer material during conjugation to other cells that do not have sex factor; the receptor cells thus acquire the capacity to be donors and in

this sense have changed their sex. Resistance transfer plasmids are characterized by genes that enable them to provide to their host cells resistance, for example, to drugs; such resistance may depend on production of an enzyme coded for by the plasmid that destroys a drug, for example, penicillinase which destroys penicillin. Bacteriocinogenic bacteria produce substances toxic to other bacteria, for example, colicins produced by plasmids of certain strains of *E. coli.* In any case, the bacterial plasmid can exist free in the cytoplasm of the cell where it reproduces autonomously, or it is occasionally inserted into the bacterial chromosome where it replicates only when the latter does.

A striking similarity between a plasmid and the coliphage lambda has been noted by Hayes (1968, Chap. 24). Plasmids and phage lambda have DNA genomes of about the same size, and both genomes are independent of the bacterial chromosome in the sense that they can replicate autonomously and if lost from the cell can be reacquired only by infection. Infection by lambda is by means of the injection mechanism commonly employed by phages in initiating infection; infection by plasmids is by conjugation which they often promote. Both, under appropriate conditions, can become integrated into host cell chromosome where they replicate coordinately with the host DNA. Both can mutate and both can undergo genetic recombination. The resemblance is strong enough so that Hayes thinks it logical to regard plasmids as viruses. The missing link, then, with respect to the origin of viruses is the demonstration that any plasmid originated from a bacterial chromosome. This proof is missing but the hypothesis is supported by the apparent homology between plasmid and chromosomal DNAs. This is presumably the basis for integration of some plasmids and the degree of homology observed is consistent with expectations if the plasmids had evolved through many generations from a segment of bacterial chromosome.

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