Comprehensive Virology 17

Comprehensive Virology

Edited by Heinz Fraenkel-Conrat
University of California at Berkeley

and Robert R. Wagner
University of Virginia

Volume 1: *Descriptive Catalogue of Viruses* – by Heinz Fraenkel-Conrat

Reproduction

Volume 2: *Small and Intermediate RNA Viruses* – Contributors: J.T. August, L. Eoyang, A. Siegel, V. Hariharasubramanian, L. Levintow, E. R. Pfefferkorn, D. Shapiro, and W. K. Joklik

Volume 3: *DNA Animal Viruses* – Contributors: L. Philipson, U. Lindberg, J.A. Rose, N.P. Salzman, G. Khoury, B. Moss, B. Roizman, and D. Furlong

Volume 4: *Large RNA Viruses* – Contributors: P.W. Choppin, R.W. Compans, R.R. Wagner, and J.P. Bader

Volume 7: *Bacterial DNA Viruses* – Contributors: D.T. Denhardt, D.S. Ray, and C.K. Mathews

Structure and Assembly

Volume 5: *Virions, Pseudovirions, and Intraviral Nucleic Acids* – Contributors: T.I. Tikchonenko, John T. Finch, Lionel V. Crawford, and H. Vasken Aposhian

Volume 6: *Assembly of Small RNA Viruses* – Contributors: K.E. Richards, R.C. Williams, L. Hirth, P.P. Hung, Y. Okada, T. Ohno, and R.R. Rueckert

Volume 13: *Primary, Secondary, Tertiary, and Quaternary Structures* – Contributors: G. Air, R. W. Compans, F. A. Eiserling, W. Fiers, R. V. Gilden, J. King, H.-D. Klenk, S. Oroszlan, and W. B. Wood

Regulation and Genetics

Volume 8: *Bacterial DNA Viruses* – Contributors: D. Rabussay, E. P. Geiduschek, R. A. Weisberg, S. Gottesman, M. E. Gottesman, A. Campbell, R. Calendar, J. Geisselsoder, M.G. Sunshine, E.W. Six, and B.H. Lindqvist

Volume 9: *Genetics of Animal Viruses* – Contributors: H. S. Ginsberg, C. S. H. Young, W. Eckhart, J. H. Subak-Sharpe, M. C. Timbury, C.R. Pringle, E.R. Pfefferkorn, P. D. Cooper, L. K. Cross, B. N. Fields, P. K. Vogt, M. A. Bratt, and L. E. Hightower

Volume 10: *Viral Gene Expression and Integration* – Contributors: A. J. Shatkin, A. K. Banerjee, G. W. Both, A. S. Huang, D. Baltimore, D. H. L. Bishop, W. Doerfler, and H. Hanafusa

Volume 11: *Plant Viruses* – Contributors: L. Van Vloten-Doting, E. M. J. Jaspars, G. Bruening, J. G. Atabekov, H. Fraenkel-Conrat, M. Salvato, L. Hirth, I. Takebe, T. O. Diener, and A. Hadidi

Additional Topics

Volume 12: *Newly Characterized Protist and Invertebrate Viruses* – Contributors: T. W. Tinsley, K. A. Harrap, K. N. Saksena, P. A. Lemke, L. A. Sherman, R. M. Brown, Jr., T. I. Tikchonenko, and L. Mindich

Volume 14: *Newly Characterized Vertebrate Viruses* – Contributors: P.E. McAllister, F.L. Schaffer, D.W. Verwoerd, A. Granoff, W.S. Robinson, J.A. Robb, D.H.L. Bishop, and W.E. Rawls

Volume 17: *Methods Used in the Study of Viruses* – Contributors: Max A. Lauffer, Harold W. Fisher, Bernard Jacrot, M. H. V. Van Regenmortel, H. Fraenkel-Conrat, Terence M. Murphy, Milton P. Gordon, and Rufus S. Day

Virus-Host Interactions

Volume 15: *Immunity to Viruses* – Contributors: M. B. A. Oldstone, B. Mandel, N. R. Cooper, R. M. Zinkernagel, E. De Maeyer, and J. De Maeyer-Guignard

Volume 16: *Viral Invasion, Persistence, and Disease* – Contributors: C. Howe, J. E. Coward, T. W. Fenger, Julius S. Youngner, Olivia T. Preble, John J. Holland, S. Ian Kennedy, Bert L. Semler, Charlotte L. Jones, Laurent Roux, Elizabeth A. Grabau, Fred Rapp, David H. Porter, H. J. Cho, Jerry S. Wolinsky, Richard T. Johnson, and R. E. F. Matthews

Comprehensive

Edited by

Heinz Fraenkel-Conrat

Department of Molecular Biology and Virus Laboratory
University of California, Berkeley, California

and

Robert R. Wagner

Department of Microbiology
University of Virginia, Charlottesville, Virginia

Virology

17

Methods Used in the Study of Viruses

PLENUM PRESS • NEW YORK AND LONDON

Library of Congress Cataloging in Publication Data

Main entry under title:

Methods used in the Study of viruses.

(Comprehensive virology; v. 17)
Includes index.
1. Virology – Technique. I. *Fraenkel-Conrat,* Heinz, 1910- II. Wagner, Robert R., 1923- III. Series: Fraenkel-Conrat, Heinz, 1910- Comprehensive virology; v. 17.
QR357.F72 vol. 17 [QR385] 576'.64s [576'.64'028] 80-21841
ISBN-13: 978-1-4615-6695-3 e-ISBN-13: 978-1-4615-6693-9
DOI: 10.1007/978-1-4615-6693-9

Softcover reprint of the hardcover 1st edition 1981
A Division of Plenum Publishing Corporation
227 West 17th Street, New York, N.Y. 10011

Foreword

The time seems ripe for a critical compendium of that segment of the biological universe we call viruses. Virology, as a science, having passed only recently through its descriptive phase of naming and numbering, has probably reached that stage at which relatively few new—truly new—viruses will be discovered. Triggered by the intellectual probes and techniques of molecular biology, genetics, biochemical cytology, and high resolution microscopy and spectroscopy, the field has experienced a genuine information explosion.

Few serious attempts have been made to chronicle these events. This comprehensive series, which will comprise some 6000 pages in a total of about 18 volumes, represents a commitment by a large group of active investigators to analyze, digest, and expostulate on the great mass of data relating to viruses, much of which is now amorphous and disjointed, and scattered throughout a wide literature. In this way, we hope to place the entire field in perspective, and to develop an invaluable reference and sourcebook for researchers and students at all levels.

This series is designed as a continuum that can be entered anywhere, but which also provides a logical progression of developing facts and integrated concepts.

Volume 1 contains an alphabetical catalogue of almost all viruses of vertebrates, insects, plants, and protists, describing them in general terms. Volumes 2–4 deal primarily, but not exclusively, with the processes of infection and reproduction of the major groups of viruses in their hosts. Volume 2 deals with the simple RNA viruses of bacteria, plants, and animals; the togaviruses (formerly called arboviruses), which share with these only the feature that the virion's RNA is able to act as messenger RNA in the host cell; and the reoviruses of animals and plants, which all share several structurally singular features, the most important being the double-strandedness of their multiple RNA molecules.

Volume 3 addresses itself to the reproduction of all DNA-contain-

ing viruses of vertebrates, encompassing the smallest and the largest viruses known. The reproduction of the larger and more complex RNA viruses is the subject matter of Volume 4. These viruses share the property of being enclosed in lipoprotein membranes, as do the togaviruses included in Volume 2. They share as a group, along with the reoviruses, the presence of polymerase enzymes in their virions to satisfy the need for their RNA to become transcribed before it can serve messenger functions.

Volumes 5 and 6 represent the first in a series that focuses primarily on the structure and assembly of virus particles. Volume 5 is devoted to general structural principles involving the relationship and specificity of interaction of viral capsid proteins and their nucleic acids, or host nucleic acids. It deals primarily with helical and the simpler isometric viruses, as well as with the relationship of nucleic acid to protein shell in the T-even phages. Volume 6 is concerned with the structure of the picornaviruses, and with the reconstitution of plant and bacterial RNA viruses.

Volumes 7 and 8 deal with the DNA bacteriophages. Volume 7 concludes the series of volumes on the reproduction of viruses (Volumes 2–4 and Volume 7) and deals particularly with the single- and double-stranded virulent bacteriophages.

Volume 8, the first of the series on regulation and gentics of viruses, covers the biological properties of the lysogenic and defective phages, the phage-satellite system P 2–P 4, and in-depth discussion of the regulatory principles governing the development of selected lytic phages.

Volume 9 provides a truly comprehensive analysis of the genetics of all animal viruses that have been studied to date. These chapters cover the principles and methodology of mutant selection, complementation analysis, gene mapping with restriction endonucleases, etc. Volume 10 also deals with animal cells, covering transcriptional and translational regulation of viral gene expression, defective virions, and integration of tumor virus genomes into host chromosomes.

Volume 11 covers the considerable advances in the molecular understanding of new aspects of virology which have been revealed in recent years through the study of plant viruses. It covers particularly the mode of replication and translation of the multicomponent viruses and others that carry or utilize subdivided genomes; the use of protoplasts in such studies is authoritatively reviewed, as well as the nature of viroids, the smallest replicatable pathogens. Volume 12 deals with special groups of viruses of protists and invertebrates which show

properties that set them apart from the main virus families. These are the lipid-containing phages and the viruses of algae, fungi, and invertebrates.

Volume 13 contains chapters on various topics related to the structure and assembly of viruses, dealing in detail with nucleotide and amino acid sequences, as well as with particle morphology and assembly, and the structure of virus membranes and hybrid viruses. The first complete sequence of a viral RNA is represented as a multicolored foldout.

Volume 14 contains chapters on special and/or newly characterized vertebrate virus groups: bunya-, arena-, corona-, calici-, and orbiviruses, icosahedral cytoplasmic deoxyriboviruses, fish viruses, and hepatitis viruses.

Following Volume 14 is a group of volumes dealing with virus–host interactions. Volume 15 focuses on immunity to viruses; Volume 16 on viral invasion, factors controlling persistence of viruses, responses to viral infection, and certain diseases; and Volume 18 on other viral diseases and cell responses to viral infection, including cell death.

The present volume contains chapters discussing and evaluating most of the biophysical, biochemical, and serological methods used in virus research.

Our knowledge of certain viruses has advanced greatly since publication of the early volumes of *Comprehensive Virology*, and a second updated edition for each of these was considered. The editors and publisher have decided that instead of such a second edition they would approach the concept of comprehensive coverage of virology in a different manner. A series of books or groups of books, each dealing with a specific virus family *in extenso*, will be planned and edited by an eminent specialist in the respective field. This series, termed *The Viruses*, will start with three books devoted to the Herpesviridae and will be edited by B. Roizman.

Contents

Chapter 3

Structural Studies of Viruses with X-Rays and Neutrons

Bernard Jacrot

Chapter 4

Serological Methods in the Identification and Characterization of Viruses

M. H. V. Van Regenmortel

Chapter 5

Chemical Modification of Viruses

H. Fraenkel-Conrat

Chapter 6

Photobiology of RNA Viruses

Terence M. Murphy and Milton P. Gordon

Chapter 7

The Photobiology of the DNA Viruses λ, T4, and φX174

Rufus S. Day III

CHAPTER 1

Biophysical Methods in Virus Research

Max A. Lauffer

The Biophysical Laboratory
Department of Biological Sciences
University of Pittsburgh
Pittsburgh, Pennsylvania 15260

1. INTRODUCTION

All viruses are composed at least of protein and nucleic acid, and certain viruses contain other biochemical constituents as integral parts of their structure. Thus the biophysical methods used in the isolation and characterization of viruses are the same as those used by biochemists generally. It would be impossible in a chapter of reasonable length to deal with all such methods exhaustively. For that reason, an attempt will be made to limit this discussion to those most extensively used by virologists. The approach will be to explain the underlying principles involved in each method and to illustrate its use by citing examples drawn more or less randomly from the literature, including previous volumes of *Comprehensive Virology*. The purpose of the present effort is to provide readers not familiar with these methods a basis for understanding them, and perhaps some appreciation of which methods are best adapted to the solution of specific problems. However, anyone desiring to make use of any of the methods must consult more detailed specific treatments available in the literature. Certain methods, like electron microscopy and X-ray crystallography, are either so widely used or so highly technical that they deserve chapters in their own right,

written by experts in the particular field. These methods are dealt with in Chapters 2 and 3 of this volume. Treatment of electron microscopy will be limited to an elementary discussion of the physical principles involved.

Even though the method of ultrafiltration seems no longer to be extensively employed, because of its historical significance it would be inappropriate not to mention it. The very name, "virus," in common use today, is a shortened form of an earlier name, "filterable virus," a name adopted to signify that these agents are distinguished from other disease-producing agents, then referred to generally as "viruses," by virtue of the fact that they pass through filters which retain bacteria. The first recognized viruses, tobacco mosaic virus (Iwanowski, 1892; Beijerinck, 1898), foot-and-mouth disease virus (Loeffler and Frosch, 1898), and yellow fever virus (Reed, 1901), were all discovered by this method, as were many others in subsequent years. The only information provided initially was that these viruses are smaller than bacteria. Later, it was found that tobacco mosaic virus, even though it could pass a Berkefeld filter, was held back by a Livingstone porous cup (Allard, 1916). This gave some idea of the lower limit of the size of this virus. Later, a variety of filters graded for pore size became available. These were used, especially by Elford (1931) and co-workers, for the determination of the approximate sizes of many viruses. It was established by this method that different viruses possess different characteristic sizes, ranging from about 10 to 300 nm.

The first method used to obtain a virus in a crystalline form was precipitation (Stanley, 1935). Since the coats of viruses are composed largely or completely of proteins, viruses exhibit the solubility characteristics of proteins. The most common methods of altering the solubility of proteins, and therefore of viruses, are adjustment of pH or salt concentration, or the addition of organic solutes. Proteins and viruses exhibit minimum solubility near their isoelectric points. The effect of salts is complex. Usually increasing the salt concentrations at very low salt concentrations increases the solubility. This effect is called salting-in. At higher concentrations, solubility decreases on further increase in salt concentration, an effect known as salting-out. The salt concentration at which salting-out begins to predominate depends on the nature of the protein surface. Proteins with highly hydrophilic surfaces tend to salt-in even at appreciable salt concentrations, while those with highly hydrophobic surfaces begin to salt-out at relatively low salt concentrations. Furthermore, the effect of salt varies considerably with the nature of the electrolyte. The effect of adding organic solutes is also complex; some organic solutes increase the solubility of some proteins, but the

predominant effect is to decrease protein solubility. Denaturation frequency occurs when organic solutes are added. Theoretical and practical aspects of protein solubility are discussed by Haschemeyer and Haschemeyer (1973) and by Edsall and Wyman (1958). Solubility methods are still used in the purification of viruses, especially in their crystallization.

Particles the size of viruses are ideal for study by centrifugation, one of the earliest physical methods employed and one still extensively used. It was found as early as 1922 that vaccinia virus could be concentrated by centrifugation (MacCallum and Oppenheimer, 1922; Bland, 1928; Ledingham, 1931; Craigie, 1932). Rivers and his associates (Hughes *et al.*, 1935; Parker and Rivers, 1935, 1936, 1937; Parker and Smythe, 1937) extended this method to prepare appreciable amounts of purified vaccinia virus. Soon after the crystallization of tobacco mosaic virus (Stanley, 1935), the method of differential centrifugation was used to purify this virus (Wykoff, *et al.*, 1937); in subsequent years, centrifugation has been widely used to isolate and characterize viruses.

2. VISCOSITY

While centrifugation is probably the most widely used of all the physical methods in the study of viruses and their components, the results of centrifugation experiments and even centrifugation theory cannot be understood without reference to diffusion and viscosity; an understanding of diffusion depends on an understanding of viscosity. For that reason, this treatment will begin with viscosity, followed by diffusion, before centrifugation is considered. More detailed, simplified treatments of viscosity are available in the literature, for example, in Lauffer (1946, 1980) and Bull (1971).

Water and most common laboratory solvents behave in accord with Newton's law of flow. Imagine two planes, each of an area, A, oriented parallel to each other and separated by a distance, z, as illustrated in Fig. 1. If a force, F, is applied to the upper plane in the direction of the arrow while the lower plane is held stationary, liquid adjacent to the upper plane will move in the direction of the arrow with a velocity, u, and that adjacent to the bottom will have a velocity of zero. The intervening fluid can be thought of as an infinite number of infinitely thin sheets all parallel to the two planes. They will move with intermediate velocities, ranging from zero at the bottom to u at the top.

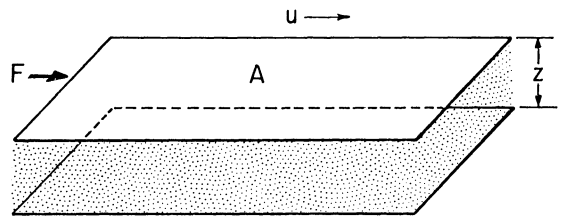


Fig. 1. Diagram illustrating Newton's law of flow. The space between the two parallel planes of area A, z cm apart, is filled with liquid of viscosity coefficient, η. The lower plane is held stationary while a force, F, applied in the direction of the arrow, causes the upper plane to move in the same direction with a steady velocity, u. Intervening liquid exhibits a velocity gradient du/dz.

Thus a velocity gradient will be established, symbolized by du/dz. This type of flow is given the name "plane laminar flow." Newton's law of flow states simply that the velocity gradient established in the fluid is directly proportional to the force per unit area applied to the upper plane. This law can be stated in equation form as

$$du/dz = (1/\eta)(F/A) \tag{1}$$

where η is the coefficient of viscosity and $1/\eta$ is the proportionality constant. In the cgs system, the unit of viscosity is the poise defined as the viscosity of a fluid in which a velocity gradient, du/dz, of 1 cm/sec/cm is produced when a force of 1 dyne is applied to an area of 1 cm^2. The dimensions of the poise are grams divided by centimeter seconds. A medium lubricating oil at room temperature has a viscosity of about 1 poise. Water at 20°C has a viscosity of almost exactly 0.01 poise. Since most biological and biochemical investigations deal with aqueous solutions, viscosity will henceforth be described in terms of the centipoise, equal to 1/100 poise. If the planes in Fig. 1 are separated by a distance of 1 cm, if the area, A, of the planes is 1 cm^2, and if the force, F, is just sufficient to produce a velocity, u, of 1 cm/sec, the velocity gradient will be 1 cm/sec/cm, the volume of liquid between the planes will be 1 cm^3, the distance moved by the upper plane will be 1 cm in 1 sec, and the work done to cause this movement will be $F \times 1$ ergs/sec. Thus the viscosity of a liquid can be thought of as the work/cm^3/sec expended in producing flow with a velocity gradient of 1 cm/sec/cm. This work is dissipated as heat.

The equation governing the flow of a liquid through a capillary tube was first discovered experimentally by Poiseuielle and was later derived from Newton's law of flow. Details of the derivation are available in many places, for example, in Tanford (1961), Bull (1971), and Lauffer (1980). When V is the volume of liquid flowing through a

capillary tube of length, l, and radius, r, in time, t, with a pressure difference across the capillary of, P, equation (2) expresses the relationship.

$$V = \frac{\pi r^4 P t}{8 \eta l} \tag{2}$$

The most commonly used apparatus for the determination of viscosity is the Ostwald viscometer, illustrated in Fig. 2. The way this viscometer is used is to measure first the time, t_0, for a fixed volume, V, of a reference liquid such as water, with viscosity coefficient of η_0 to flow through the capillary. The unknown solution is then placed in the same viscometer and the time, t, for the same volume, V, to flow is determined. From equation (2), when η is the unknown viscosity coefficient of the liquid being measured,

$$\eta/\eta_0 = tP/t_0P_0 = td/t_0d_0 \tag{3}$$

The final term on the right derives from the fact that when flow is under the influence of gravity the average pressure difference is proportional to the density, d. Since the viscosity of water is precisely known, it can be used as a reference material. With this value, the viscosity of any other liquid or solution can be determined with the aid of equation (3). The ratio η/η_0 is frequently called the relative viscosity. Viscosity varies with temperature; for example, the viscosity of water is 1.7921 centipoises at 0°C, 1.0050 at 20°C, and 0.2838 at 100°C. Tables of values at other temperatures are given in various reference volumes, for example, *Handbook of Chemistry and Physics.*

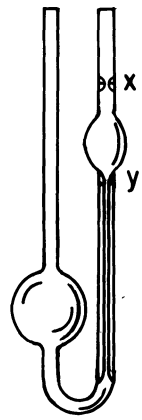


Fig. 2. Diagram of an Ostwald viscometer.

When solutes are dissolved in the solvent, the coefficient of viscosity is changed; usually it increases. The viscosities of some solutions are tabulated in handbooks as a function of concentration, but when data cannot be found the viscosity can be measured by the method just described. It is necessary to know accurately the viscosity of the medium in which macromolecules move in diffusion, in centrifugation, and in any other transport experiment in order to interpret the results.

Beyond providing such essential information, viscosity measurements can also provide useful information about macromolecules. Experience has shown that the viscosity of a solution of macromolecules depends on the volume fraction of the macromolecular component in accord with a general equation of the form

$$\frac{\eta}{\eta_0} = 1 + AG + BG^2, \text{ etc.} \tag{4}$$

where G is the volume fraction of the macromolecule and A and B are constants assigned to make the data fit the equation.

$$\frac{\eta}{\eta_0} - 1 = AG + BG^2 \tag{5}$$

$(\eta/\eta_0 - 1)$ is called the specific viscosity.

$$\left[\left(\frac{\eta}{\eta_0}\right) - 1\right]/G = A + BG \tag{6}$$

As G approaches zero,

$$\left[\left(\frac{\eta}{\eta_0}\right) - 1\right]/G = A \equiv [\eta] \tag{7}$$

The symbol $[\eta]$ is called the intrinsic viscosity; it depends on two properties of the macromolecule, hydration and shape. Intrinsic viscosities are determined experimentally by measuring the viscosities of solutions at different volume concentrations. Many ways exist to evaluate $[\eta]$ from such data, the simplest of which is to plot $[(\eta/\eta_0) - 1]$ against G and then draw a tangent to the curve at $G = 0$. The slope of this tangent is A or $[\eta]$.

To interpret further, it is necessary to have a model for the particle. The usual idealized models are ellipsoids of revolution. When an ellipse is rotated about its major axis, it generates an elongated or

prolate ellipsoid of revolution. If it is rotated about the minor axis, it generates a platelike or oblate ellipsoid of revolution. Intermediate between these is the sphere, generated by rotating an ellipse with equal axes (a circle) about any diameter. These models have been very useful in interpreting many kinds of hydrodynamic data, but it must be remembered that no real particle, virus, or protein molecule ever corresponds exactly to an idealized mathematical model. Even nearly spherical particles have surfaces with are rough at the atomic level and are therefore not strictly spherical. Nevertheless, the use of models can be informative. The simplest is the sphere.

Einstein showed that the viscosity of a suspension of spherical particles is related to the volume fraction by the equation

$$\frac{\eta}{\eta_0} = 1 + 2.5G \tag{8}$$

A simplified derivation of this equation has been achieved recently (Lauffer, 1980). From this equation and from equation (7), it follows that $[\eta]$ for spheres is 2.5. If the spheres are hydrated such that the hydrated volume of each sphere is K times the unhydrated volume, then the hydrodynamically effective volume fraction of the sphere will be KG. Since in any experiment the analytically determined value of the volume fraction will be that of unhydrated particle, G, this is the quantity which will be used to calculate the intrinsic viscosity. Thus the experimental value of the intrinsic viscosity of hydrated spheres will be $2.5K$. When it is known that the macromolecule can be represented reasonably accurately by a spherical model, the intrinsic viscosity makes it possible to evaluate K. Intrinsic viscosity data assembled from the literature yield values of K of 2.51 and 3.27 for southern bean mosaic virus and rabbit papilloma virus, respectively (Lauffer and Bendet, 1954).

The reason that macromolecules increase the viscosity of a solution is that, in a velocity gradient, the macromolecule moves with a velocity equal to that of liquid at its midplane, but it extends into regions where the liquid is flowing faster than and slower than the midplane. Thus liquid must flow around the particle, dissipating energy. This added dissipation of energy increases the viscosity. When the particle is a rod-shaped ellipsoid or a platelike ellipsoid with long axis oriented perpendicular to the direction of flow, it extends into regions where the flow rate of the liquid differs from that at the midplane to a greater extent than would be the case if the particle were a sphere of the same volume.

There are two consequences. One is that the dissipation of energy, and therefore the viscosity contribution, is greater than it would be if the particle were a sphere, and the other is that a torque is applied to the particle, causing it to rotate. This torque is at a maximum when the particle is oriented with its long axis perpendicular to the direction of flow and at a minimum when the particle is parallel to the direction of flow but never zero. The result is a type of rotation described as precessional, which leads to a tendency for the particle to orient with its long dimension in the direction of flow. Superimposed on this hydrodynamic orientation is the disorienting tendency stemming from random thermal rotation. All of these effects have been taken into account theoretically by Simha (1940), who derived equations for the intrinsic viscosity of randomly oriented ellipsoids of revolution, both prolate and oblate. Simha's result can be expressed by equations of the generalized form,

$$[\eta] = K\Psi(b/a) \tag{9}$$

where b is the major and a the minor semiaxis of the ellipsoid and $\Psi(b/a)$ should be read as "function of b/a." This function is presented in brief numerical form in Table 1. More detailed tables are available in many publications, for example, in Schachman (1959). A simple way to solve $\Psi(b/a)$ in terms of b/a is to plot it against b/a for both prolate and oblate ellipsoids and to connect the points with a smooth curve. This can be used to evaluate b/a for measured values of $[\eta]/K$.

3. DIFFUSION

3.1. Theory

To understand the theory and practice of centrifugation and to interpret some of the results, it is necessary to understand diffusion. The basic law for the diffusion of solute molecules through a solvent in a single direction was discovered by Fick and is presented below in two alternative forms, Fick's first law, equation (10), and Fick's second law, equation (11):

$$dS = -DQ(dc/dx)dt \tag{10}$$

$$dc/dt = D\, d^2c/dx^2 \tag{11}$$

TABLE 1

Numerical Solution of Simha's Equations

	$\psi(b/a) = [\eta]/K$	
b/a	Prolate ellipsoids (elongated)	Oblate ellipsoids (flattened)
1	2.50	2.50
2	2.91	2.85
5	5.81	4.71
10	13.63	8.04
20	38.6	14.8
50	176.5	35.0
100	593.0	68.6

In these equations, dS is the amount of material diffusing through a cross-section, Q, in time dt, when the concentration gradient is dc/dx. D is the diffusion coefficient with dimensions of cm^2/sec in the cgs system. The two equations are aspects of the same law in the sense that one can be derived from the other. Details are provided in many references, for example, Tanford (1961), and Haschemeyer and Haschmeyer (1973). Diffusion coefficients are funtions of the size and shape of the diffusing particle, but they also depend on temperature. The nature of that dependence is made clear by the Einstein-Sutherland equation:

$$D = \frac{RT}{Nf} \tag{12}$$

R is the gas constant, T is the absolute temperature, N is Avogadro's number, and f is the coefficient of friction. When a particle moves through a viscous medium with a velocity u, it encounters a force of resistance proportional to u. The proportionally constant is f. For spheres, it is given by Stokes's law.

$$f = 6\pi\eta r \tag{13}$$

A simplified derivation of Stokes's law has been worked out (Lauffer, 1980). When equation (13) is substituted into equation (12),

$$D = RT/6\pi rN\eta \tag{14}$$

Stokes's law cannot be used to give the value of f for nonspherical particles, but f is still proportional to η, which, in turn, depends on T and on the nature of the solvent. It is, therefore, necessary to convert measured values of D to some standard condition in order to provide comparative values from solute to solute. The commonly agreed-on standard system is to evaluate the diffusion coefficient the particle would have in a medium with the viscosity of water at 20°C, symbolized by D_w^{20}. From equation (14) one obtains,

$$D_w^{20} = D \frac{293.16\eta}{T\eta_w^{20}} \tag{15}$$

where D is the diffusion coefficient measured at temperature T in a medium with viscosity coefficient η and η_w^{20} is the viscosity of water at 20°C.

3.2. Porous Disk Method

The simplest method of measuring D is with the Northrop-Anson (1929) diffusion cell, illustrated in Fig. 3. This cell consists of a coarse glass porous disk or filter fused to the end of a cup. The cup is filled with the solution to be analyzed and must be closed to prevent flow through the disk. It is then immersed in the solvent and allowed to stand for an appropriate time at constant temperature. Equation 10 can be used to evaluate D because a constant concentration gradient (dc/dx) is established across the filter equal to $(c_i - c_0)/l$, where c_i and c_0 are the concentration inside and outside, respectively, and l is the thick-

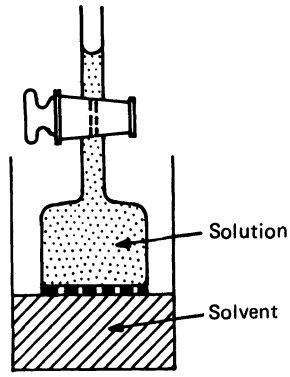


Fig. 3. Diagram of the porous-disk diffusion apparatus.

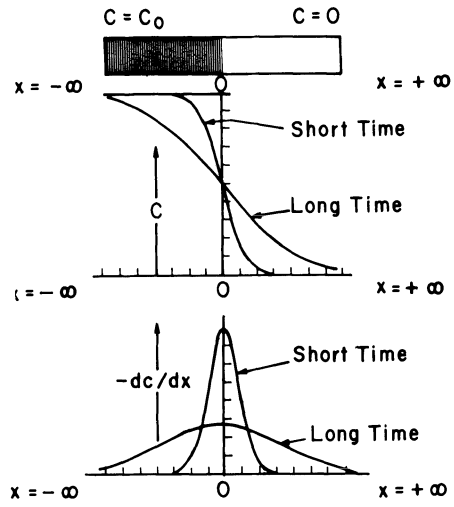


Fig. 4. Free diffusion at an interface. Top: Concentration of solute initially. Center: Concentration, c, plotted as a function of position, x, after two times of diffusion. Bottom: Concentration gradient as a function of position after same two times.

ness of the filter. When the experimental arrangement is properly controlled, c_i is always practically equal to the loading concentration, c, and c_0 is practically equal to zero. This permits integration of equation (10) by merely substituting ΔS and Δt for dS and dt. The amount, ΔS, which diffuses out in the time of the experiment, Δt, can be determined by multiplying the concentration after mixing in the external vessel by the volume of solvent in it. Thus D is determined from a ratio of concentrations. It is not necessary that an absolute method of concentration measurement be available; relative values suffice, but it is necessary for them to be accurate in order to obtain an accurate value for D. Bioassays can be used, provided that they are sufficiently accurate. The limitation of the method stems from the fact that Q in the equation depends on the average cross-section of the pores in the filter. The normal way of using the cell is to determine the apparatus constant, Q/l, by carrying out a diffusion experiment on material with known diffusion coefficient, D. This value of Q/l can then be used in subsequent experiments. This method is so simple that it is surprising that it is not used extensively in virus research.

3.3. Free Diffusion

The most frequently used method of measuring diffusion coefficients involves free diffusion at an interface. As is illustrated in Fig. 4, a very sharp boundary is established between a solution and a solvent. Normally, the solution is at the bottom of the vessel because solutions of most biological materials are more dense than the usual solvent,

water. The system with its boundary is then maintained at an accurrately controlled temperature for as long as the experiment requires. The solute molecules or particles gradually diffuse into the solvent, so that at the end of time t there is a sigmoidal change of concentration from deep in the solution to high in the solvent, as illustrated in Fig. 4. This is in contrast with the sharp break in concentration at the boundary at the beginning of the experiment, when t was zero. In representing this system mathematically, it is most convenient to let x equal 0 at the position of the initial sharp boundary, $-\infty$ deep in the solution and $+\infty$ high in the solvent. While this change in concentration can be measured by chemical or biological assay on samples, it is usually determined by optical means. The most frequently employed optical systems are based on the schlieren principle. Such methods measure the refractive index gradient, dn/dx, as a function of position, x. For dilute solutions, the refractive index, n, is related to the concentration, c, by

$$n = n_0 + kc \tag{16}$$

In this equation, n_0 is the refractive index of the solvent and k is the specific refractive increment of the solute in that solvent. When equation (16) is differentiated, equation (17) is obtained:

$$dn/dx = k\, dc/dx \tag{17}$$

Thus the refractive index gradients measured by optical methods based on the schlieren system are directly proportional to concentration gradients. Inspection of Fig. 4 shows how dc/dx varies with distance: dc/dx is zero deep in the solution where the concentration remains constant at its initial value, c_0, and also high in the solvent where the concentration is zero. It has the maximum negative value at $x = 0$, where the concentration change is steepest.

To interpret diffusion experiments of this sort, it is necessary to start with Fick's second law, equation (11). This can be integrated to give equation (18). The simplest method of carrying out this integration has been published by Longsworth (1945).

$$dc/dx = -\frac{c_0}{\sqrt{4\pi Dt}}\, e^{-x^2/4Dt} \tag{18}$$

Equation (18) is the equation for a bell-shaped curve like that shown in Fig. 4. Inspection of the equation shows that when x is either $+\infty$ or

$-\infty$, dc/dx is zero, and when x is zero, dc/dx has its greatest negative value, $-c_0/\sqrt{4\pi DT}$. Since all variables in the equation can be measured, D can be determined. Various convenient methods of evaluating D from curves such as those shown in Fig. 4 are available and have been described on many previous occasions, for example, by Neurath (1942).

Diffusion coefficients can also be determined from free diffusion experiments by measuring concentration, c, as a function of position, x. Analyses on samples withdrawn from different positions would give such data. Futhermore, some optical methods, particularly those based on the interference principle or on optical absorption, give the data in this form. To complete the interpretation, equation (18) must be integrated once more. This integration can be carried out by substituting z for $x/\sqrt{2DT}$.

$$z = x/\sqrt{2DT} \tag{19}$$

When this is done, equation (18) takes the form of

$$dc = -\frac{c_0 e^{-z^2/2}}{\sqrt{2\pi}}\, dz \tag{20}$$

When equation (20) is integrated between z, where the concentration is c, and ∞, where the concentration is zero, equation (21) is obtained.

$$\frac{c}{c_0} = \frac{1}{\sqrt{2\pi}} \int_z^{\infty} e^{-z^2/2}\, dz \tag{21}$$

The integral on the right cannot be solved analytically, that is, it cannot be expressed in terms of a simple equation. It is the integral commonly used in statistical theory, and tables are available which give values for various values of z. Almost any textbook on statistics will provide such tables, but they are also available in *Handbook of Chemistry and Physics*. These tables can be used to evaluate z in the following way. The analyses will give the value of c at some particular value of x. Since c_0 is known, the value of the integral, equal to c/c_0, at the value of x is also known. From the tables, one then finds the value of z corresponding to that value of the integral. Since x and z are both now known, D can be determined from equation (19).

There is another method, similar in principle but different in detail, also commonly used to solve equation (18). It involves substituting y for

$x/\sqrt{4DT}$. This yields ultimately an equation somewhat like equation (21), but different in detail. This method is called the error-function method. Tables are available for the error function; these tables are different from the ones described above. Whichever of these alternatives one chooses to use, it is necessary to recognize that they are slightly different and that the appropriate tables must be consulted; error-function tables cannot be used with equation (19).

3.4. Gel Diffusion

Diffusion experiments can also be carried out in gels like gelatin or agaragar. For the purpose of understanding diffusion, agaragar or gelatin gels can be thought of as irregular networks of fibrous gelatin or agaragar, perhaps swollen by hydration, with large amounts of solvent entrapped. As long as the regions filled with intact solvent are large enough so that the macromolecular solute is not retained as in a filter, diffusion can take place through the gel. However, the solute molecules must move around the fibers in their path. This has the effect of increasing the path length as it moves forward a net distance of x. The theory of diffusion in gels has been discussed at some length (Lauffer, 1961). Equations (10) and (11) and all equations derived from them must be altered in two ways. First, D must be replaced by D', defined by

$$D' = D \frac{1}{1 + (\alpha - 1)\phi} \tag{22}$$

For randomly oriented cylinders or threads such as one might assume a gelatin or agaragar gel to have, α has a value of 5/3. In this equation, ϕ is the volume fraction of the gel substance, that is, the fraction of the total volume of the gel occupied by the swollen fibers in the network, really, the volume fraction from which solute molecules are excluded. The other change is that c must be replaced by c', defined as

$$c' = c(1 - \phi) \tag{23}$$

This has the effect of changing concentration in amount per unit volume of liquid to amount per unit volume of gel.

A simple way of determining diffusion coefficients using gels is to place a solution of macromolecules on top of a layer of gel in a petri dish. After time t the total amount of solute which has diffused into the

gel, ΔS, is determined by any convenient method of analysis. ΔS is related to D' by (Lauffer, 1961)

$$\Delta S = \frac{2Qc_0'\sqrt{D't}}{\sqrt{\pi}} \tag{24}$$

In this equation, Q is the area of the surface of the gel. c_0' is determined from c_0 by using equation 23. The value of D' obtained can then be converted to the appropriate value of D by using equation (22). Another method of measuring diffusion coefficients in gels is to allow diffusion to take place in a cylinder of gel, which is later sliced. An approximate apparatus is illustrated in Fig. 5. The concentration in each slice is then determined. This method was used by Schantz and Lauffer (1962) to demonstrate that diffusion coefficients determined in this manner agree with those obtained by other methods. They also used the method to

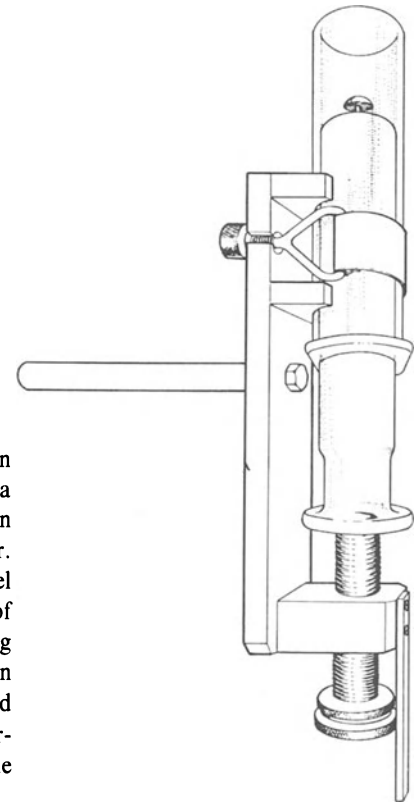


Fig. 5. Apparatus for measuring diffusion in a gel. The essentials of the apparatus are a 25-ml syringe with the bottom cut off and an accurate screw for advancing the plunger. The open space at the top is filled with gel and then immersed in the solution. At end of the experiment, gel is extracted by turning the screw and is then cut into slices of known thickness for analysis. From Schantz and Lauffer (1962). Reproduced with the permission of the copyright owner, the American Chemical Society.

determine the diffusion coefficient of tobacco mosaic virus protein in very dilute solution. A value of 9×10^{-7}cm^2/sec was obtained.

3.5. Immunodiffusion

A widely used application of diffusion in gels is the method of immunodiffusion, a method to which immunochemists can rightfully lay claim. Soluble antigens form precipitates with their specific antibodies when the concentrations of antigen and antibody are equivalent. This is known as the precipitin reaction. However, when either antibody or antigen is in substantial excess, no precipitate results. An explanation sufficient for present purposes is that the specific antibody can be considered to be bivalent and the antigen polyvalent. When the numbers of antigen and antibody molecules are equivalent, large threedimensional networks can be formed, which precipitate. However, if antibody is present in great excess, each antigen molecule will be saturated with antibody and there will be no antigen surface for the free ends of the antibodies to join. Similarly, when antigen is present in great excess, both valence sites of each antibody molecules will be joined to antigen and there will be no free antibody sites left for the uncombined antigen sites to join. In neither case will networks be formed; there will be no precipitates. In actual practice, antigen–antibody equivalance, where precipitates are formed, is represented by ranges of concentrations rather than by sharp values.

The method of immunodiffusion was developed by Oudin (1952) and extended by Ouchterlony (1958). If a solution containing an antigen is placed on top of a column of gel containing the specific antibody, the antigen will diffuse into the gel. When the concentration builds up to the range of antigen–antibody equivalence, a precipitate will form. This will show up as a turbid disk in the gel, as illustrated in Fig. 6. However, as diffusion continues, the concentration of the antigen at the initial position of the disk will increase and that of the antibody might decrease because of its diffusion into the solvent above. The range of antigen–antibody equivalence will eventually be exceeded, and the precipitate will dissolve. However, farther down in the tube, a new zone of antigen–antibody equivalence will be formed. Thus the disk tends to migrate with time. If more than one antigen is present in a given sample and if antibodies for all of the antigens have been developed in immunized animals, then a number of disks will be produced equal to the number of antigens and antibodies present, provided only that the concentrations and the diffusion coefficients permit resolution.

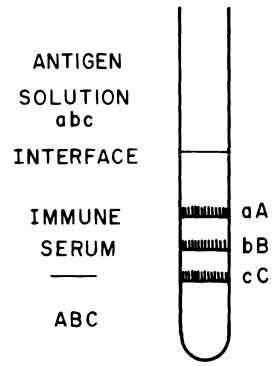


Fig. 6. Immunodiffusion by the method of Oudin.

A modification of this method is to cut out wells in a gel in a petri dish. Antigens and antibodies can then be placed in different wells and diffusion can take place. In the region where a specific antigen meets its antibody at concentrations in the equivalence zone, a curved line of precipitate will form. This is illustrated in Fig. 7. The application of the method in the field of plant virology is discussed by Ball (1964). This method is widely used to establish the relationship between viruses, illustrated by the results on TMV (Matthews, 1970) shown in Fig. 8.

Chapter 4 deals in detail with the serology of viruses.

4. CENTRIFUGATION

4.1. Theory

If an object is twirled on the end of a string, it will be constrained from flying off tangentially by a force in the string directed toward the

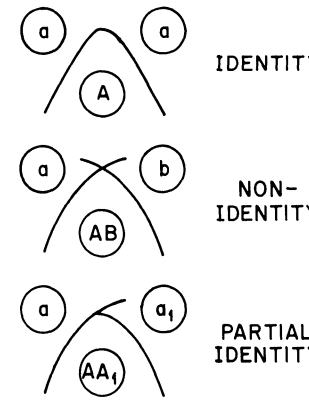


Fig. 7. Diagram of Ouchterlony method of immunodiffusion. Antibodies (capital letters) and antigens (small letters) are placed in holes cut in a suitable gel. Precipitates are formed where antigen meets homologous antibody.

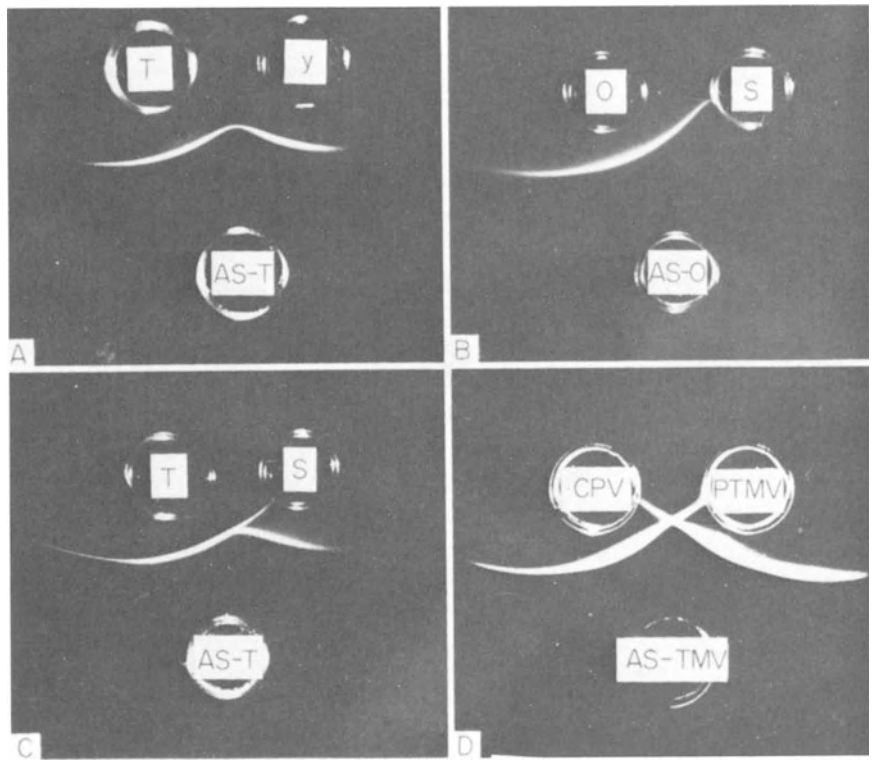


Fig. 8. Types of pattern found in immunodiffusion tests illustrated by the reactions of some strains of TMV. A: Pattern of coalescence or fusion. Antigens: T, TMV, common strain; y, a yellow mutant derived from the common strain. Antiserum (AS) to TMV. The fusion of the lines shows that the antigens compared are very similar if not identical serologically. B: Failure of an antiserum to give a visible heterologous reaction. Antigens: O, *Odontoglossum* ringspot virus; S, Sammons's *Opuntia* virus. Antiserum (AS) to O. (Both viruses react with TMV antiserum.) C: Pattern of partial intersection showing the formation of a spur. Antigens: T, TMV; S, Sammons's *Opuntia* virus antiserum (AS) to TMV. This pattern demonstrates that not all antibodies in the TMV antiserum are bound in the precipitation line formed with S antgen. Unbound antibodies diffuse through this line and form a spur with T antigen. D: Double spur formation. CPV, bean form of TMV; PTMV, U2 TMV; AS-TMV, antiserum against type TMV. The reaction shows that both strains combine with some of the same antibodies in the TMV type antiserum. Each strain also combines with antibodies that do not react with the other strain (Matthews, 1970). Reproduced with the permission of the copyright owner, Academic Press, Inc.

axis of rotation. This is called a centripetal force. Its magnitude is the mass of the object, m, times the acceleration toward the axis of rotation, $\omega^2 x$, where x is the distance along the string from the axis of rotation and ω is the angular velocity in radians per second, which, in turn, is 2π times the number of revolutions per second. When a particle in suspension in a liquid is centrifuged, it is also constrained from flying off tangentially by a centripetal force directed toward the axis. Since the particle is not rigidly attached, it actually sediments along a radius in the liquid with a velocity, v, outward if the particle is more dense than the liquid. This motion generates a force of resistance equal to the coefficient of friction, f, times v. This is one expression for the centripetal force. A second expression is the product of the acceleration and the effective mass. In this case, a buoyancy correction must be made. The effective mass is the mass of the particle minus the mass of the liquid displaced by the particle. In a two-component system, commonly called a binary system, the mass of liquid displaced is exactly the product of the mass, m, and the partial specific volume, V, of the particle and the density, d, of the fluid. When these two expressions for the centripetal force are equated, one obtains $fv = \omega^2 xm(1 - Vd)$. When the velocity is expressed as dx/dt instead of v, the equation can be arranged to give

$$s = (1/\omega^2 x)(dx/dt) = m(1 - Vd)/f \tag{25}$$

The expression in the center is the velocity of sedimentation per unit of acceleration, commonly called the sedimentation coefficient and represented by the symbol s, with dimensions of time. Since most materials of interest have sedimentation coefficients between 1 and 1000×10^{-13} sec, the commonly accepted unit of sedimentation is defined as 10^{-13} sec and is called the svedberg in honor of The Svedberg, who perfected the technique of ultracentrifugation half a century ago. Thus a virus with a sedimentation coefficient of 200×10^{-13} sec is said to have a sedimentation coefficient of 200 svedbergs, represented as 200 S.

As was discussed before, for particles of any shape, f is proportional to η. Thus the sedimentation coefficient is always inversely proportional to η, regardless of particle shape. It is conventional to consider η to be the viscosity of the suspension medium, that is, the buffer or the sucrose solution in which the particles are suspended. When this is done, it frequently turns out that the sedimentation coefficient is lower in concentrated solutions or suspensions of proteins or viruses

than in infinitely dilute ones. A practical solution to this problem is to extrapolate coefficients to infinite dilution. However, it was pointed out many years ago, especially for the case of viruses, that a sedimentation coefficient independent of concentration of the virus is obtained when η is taken as the viscosity coefficient of the actual virus solution (Lauffer, 1944*b*).

Since both the viscosity, η, and the density, d, depend on the temperature and on the composition of the medium, the sedimentation coefficient, s, likewise depends on such variables. To have a value which can be compared with values of other materials, it is necessary to correct the sedimentation coefficient to some standard condition. The conventional choice is a medium with the viscosity and density of water at 20°C. Sedimentation coefficients corrected in this manner are represented by the symbol s_w^{20}. They are obtained from actual sedimentation coefficients in experiments, s, in media of density d and viscosity η by applying the following formula:

$$s_w^{20} = \frac{s(1 - Vd_w^{20})\eta}{(1 - Vd)\eta_w^{20}} \tag{26}$$

The partial specific volume, V, is defined exactly as the partial derivative of the volume of a solution with respect to mass of solute added. It is almost exactly approximated by the ratio of the volume increase in a solution to the mass of solute added when a small mass is added to a large volume of solution. It is expressed in terms of ml/g. When the volumes of solution and solute are strictly additive, the partial specific volume is equal to the specific volume, which in turn is equal to the reciprocal of the density of the added material. In the usual case, however, there are interactions between solute and solvent which cause volumes to be nonadditive. Then the partial specific volume differs, sometimes slightly and sometimes greatly, from the reciprocal of the dry density. Furthermore, in the general case, partial specific volumes vary with concentration of the solution to which more solute is added. In spite of all of this, the usual practice, especially in the field of virology, is to use in place of the partial specific volume an apparent partial specific volume, a kind of average obtained directly by measuring the density of a solution of the virus. The appropriate formula is

$$V = \frac{m_1}{m_2}\left(\frac{1}{d} - \frac{1}{d_1}\right) + \frac{1}{d} \tag{27}$$

where m_1 and d_1 are the mass and the density of the solvent, m_2 is the mass of solute added, and d is the density of the resultant solution. Whenever the partial specific volume does not vary greatly with concentration, the apparent partial specific volume thus determined is a satisfactory substitute for the partial specific volume. Usually it is the only value available.

In binary systems, the expression $(1 - Vd)$ is the proper buoyancy correction, even for solvated or hydrated particles. The reason is that any associated water has an effective mass of zero when in a solvent of pure water. Many experiments on viruses and other biological materials, however, are carried out in systems which differ greatly from binary solutions. Examples are sedimentation in sucrose and in cesium chloride solutions. In such cases, any water associated with the biological particle no longer has zero effective mass because the partial specific volume of water multiplied by the density of the solution is no longer 1. For such experiments, the appropriate specific volume to substitute for the partial specific volume in making the buoyancy correction is V_h. The definition is

$$V_h = \frac{m_2 V_2 + m_h V_1}{m_2 + m_h} = \frac{V_2 + (m_h/m_2)V_1}{1 + (m_h/m_2)} \tag{28}$$

In this formula, m_h/m_2 is the mass of water of hydration associated with unit mass of solute, V_2 is the partial specific volume of solute, and V_1 is the partial specific volume of water, practically equal to 1 ml/g.

Most viruses and components of viruses studied by the method of centrifugation are electrically charged at the pH values at which they are studied. This leads to a possible error in the sedimentation coefficient. The simplest explanation is that in the centrifuge the charged virion or protein molecule sediments much more rapidly than the oppositely charged small ions obtained when the macromolecule ionizes. This sets up a potential gradient which acts on the charged macromolecule to impose an electrophoretic force. Similarly, positively and negatively charged small ions in the solution usually have different although small sedimentation coefficients. This also tends to establish potential gradients which act on the charged macromolecule. The first of these two effects is usually the larger; it always results in a velocity of sedimentation less than that which the macromolecule would have if it were uncharged. The second effect can either increase or decrease the sedimentation velocity, depending on whether the small ions with charge of the same sign or of the opposite sign to the macromolecule

sediment faster. While these charge effects in sedimentation are of intrinsic interest to theorists, they are to students of macromolecules primarily errors to be avoided. An obvious way to suppress both charge effects is to reduce the net charge on the macromolecule as much as possible by choosing a pH value at or near the isoelectric point. This is not always possible, because many biological macromolecules are insoluble at the isoelectric point. A totally satisfactory way of suppressing the major contribution to the charge effect is to increase the conductivity of the solution in which the experiment is done. This decreases the potential gradient and therefore the electrokinetic force acting on the macromolecule. Experience has shown that salt concentrations of approximately 0.1 M suffice to render this charge effect negligible. The effect resulting from the unequal sedimentation of small ions can be reduced to a negligible level by choosing a neutral salt with positive and negative ions with approximately equal sedimentation coefficients. KCl is the best choice for this purpose. However, it is usually necessary to control the pH of the system with a buffer. This makes it impractical to use KCl as the sole electrolyte. Charge errors are encountered in both diffusion and viscosity experiments, attributable in the main to somewhat similar causes. The remedy is basically the same as in sedimentation; approximately 0.1 M electrolyte suffices to reduce the errors to negligible values. Thus it is that well-designed sedimentation, diffusion, and viscosity experiments on charged macromolecules are carried out in solutions containing adequate amounts of low-molecular-weight electrolytes.

4.2. Interpretation of Sedimentation Coefficients

Sedimentation coefficients are determined in the ultracentrifuge. The history of the development of the ultracentrifuge and of early applications in the studies of proteins and viruses is described in great detail by Svedberg and Pedersen (1940). More recent ultracentrifugation techniques, with special emphasis on applications in biochemistry, are dealt with authoritatively by Schachman (1959). The reader interested in experimental details is referred to these sources. The discussion here will be confined to ways in which sedimentation coefficients can be helpful in the study of viruses and components of viruses.

Equation (25) shows that when the partial specific volume is known the sedimentation coefficient is a direct measure of the ratio of mass-to-friction coefficient. The mass of an unhydrated sphere is $4/3\pi r^3 d_2$, where d_2 is the actual density of the particle. For those

systems in which the partial specific or the apparent partial specific volume is actually equal to the reciprocal of the density, that is, for solutes and solvents in which the volumes are strictly additive, d_2 can be replaced by $1/V$. When the particle is an unhydrated sphere, the r in Stokes's law is the unhydrated radius. By substituting equation (13) into equation (25) and solving for r^2, one obtains

$$r^2 = \frac{9\eta s V}{2(1 - Vd)} \tag{29}$$

However, strictly unhydrated particles are rarely encountered in biological investigations. If the particle can be represented by a hydrated sphere, there is an additional problem associated with the interpretation of equation (25). The m in that equation refers to the unhydrated mass, which is related to the radius the particle would have if it were unhydrated. The friction coefficient, f, is related by Stokes's law to the hydrated radius. An accurate solution in terms of the radius of either the unhydrated or the hydrated particle is not possible without additional information concerning the extent of hydration. This dilemma is solved by eliminating f from equation (25) by substituting the Einstein-Sutherland equation, equation (12). The Svedberg equation is thus obtained.

$$M = \frac{RTs_w^{20}}{D_w^{20}(1 - Vd)} \tag{30}$$

The M in this equation is the anhydrous molecular weight because it was obtained by multiplying the anhydrous mass, m, by Avogadro's number, N. With the Svedberg equation, one can obtain the anhydrous molecular weight of particles of any shape and of any degree of hydration. In this manner, the virion of tobacco mosaic virus (Lauffer, 1944*a*) was found to have a mass of approximately 4×10^7 daltons, that of tomato bushy stunt virus (Neurath and Cooper, 1940) approximately 10×10^6, and that of the southern bean mosaic virus (Miller and Price, 1946) approximately 7×10^6.

Considerably more information can be obtained from measurements of s and D. Since the mass of unhydrated spheres can be given by $(4/3)\pi r_0^2/V$, the radius, r_0, that a particle of mass M/N would have if it were an unhydrated sphere can be calculated. When this is substituted into equation (13), the friction coefficient, f_0, that the particle would have if it were an unhydrated sphere can be calculated. The actual coefficient

of friction, f, of the particle, assumed to be a hydrated ellipsoid of revolution, can be obtained directly from the measurement of D through equation (12). Thus a friction ratio, f/f_0, can be obtained experimentally. If the particle were a hydrated sphere with a volume K times the unhydrated volume, its hydrated radius would be $K^{1/3}$ times r_0 and f would be $K^{1/3}$ times f_0.

Both Herzog and Perrin showed that the friction ratios of ellipsoids of revolution depend on the ratio of major to minor semiaxes. The combination of both of these effects can be represented by

$$f/f_0 = K^{1/3}\phi(b/a) \tag{31}$$

where $\phi(b/a)$ should be read as "function of the ratio of the major semiaxis, b, to the minor semiaxis, a, of the ellipsoid of revolution." Different functions apply for prolate and oblate ellipsoids. The best solution is to present them in tabular form as in Table 2. More detailed tables are found in Schachman (1959) and in Svedberg and Pedersen (1940). As an alternative to consulting a more detailed table, smooth curves for interpolation can be obtained by plotting $K^{-1/3}f/f_0$ against axial ratio for both prolate and oblate ellipsoids of revolution.

The use of Table 2 can be illustrated as follows. If the particle is isodimensional, that is, nearly spherical, its axial ratio is 1, and, from the table, its experimentally determined frictional ratio, f/f_0, will be $1.000K^{1/3}$. Thus the friction ratio can be interpreted directly in terms of K, the ratio of the hydrated to the unhydrated volume. In this manner, the hydrations of tomato bushy stunt virus, southern bean mosaic virus, rabbit papilloma virus, and turnip yellow mosaic virus were determined from values of s^{20}_w, D^{20}_w, and V obtained from the literature. When these

TABLE 2

Friction Ratios of Ellipsoids of Revolution

Axial ratio (b/a)	Prolate $\phi(b/a) = K^{-1/3}(f/f_0)$	Oblate $\phi(b/a) = K^{-1/3}(f/f_0)$
1	1.000	1.000
3	1.112	1.105
5	1.255	1.224
10	1.543	1.458
20	1.996	1.782
50	2.946	2.375
100	4.067	2.974

values are expressed in terms of K, they are 2.06, 2.11, 3.22, and 2.02, respectively, for the four viruses (Lauffer and Bendet, 1954).

Tobacco mosaic virus, known from the exhaustive studies to be a rodlike particle with a mass close to 40×10^6 daltons, represents the opposite extreme. Various values of s_w^{20}, D_w^{20}, and V in the literature were reviewed by Lauffer and Stevens (1968). Values of M, all in the neighborhood of 40×10^6, can be calculated, depending on which of the reported values are chosen for the experimental parameters. If the sedimentation coefficient is taken to be 187 S, the partial specific volume to be 0.73 (Lauffer, 1944*a*), and D_w^{20} to be 4.4×10^{-8} (Schramm and Bergold, 1947), the virion mass turns out to be 38.4×10^6. In the manner described above, f/f_0 can be calculated, and, with the help of Table 2, b/a can be evaluated. With M/N, V, d, and b/a known, it is a simple problem in geometry to calculate the actual dimensions of the TMV rod. This was done by Lauffer (1944*a*) with results in reasonable agreement with those obtained from electron microscopy, thus constituting a good experimental verification of the validity of the theoretical functions, $\phi(b/a)$. Earlier calculations (Neurath and Saum, 1938) of the same sort yielded values somewhat in error because D was taken to be about 3×10^{-8} instead of 4.4×10^{-8}. In this particular case, the value of b obtained is not particularly sensitive to the value of K chosen because hydration probably affects a only and not b.

Any method of evaluating f can be used to solve equation (25). One method is of historic significance because it provided the first indication of the approximate dimensions and weight of the TMV particle before the electron microscope was generally available (Lauffer, 1938). The equations of Simha (1940), equation (9), make it possible to evaluate the viscosity of solutions or suspensions of ellipsoids of revolution in terms of the axial ratio, b/a. At the time the first work on TMV was done, the Simha equations were not available, but others giving nearly the same result were. When b/a is known, f/f_0 can be obtained through the use of Table 2. Since f is equal to $f_0(f/f_0)$, and since f_0 is related by equation (13) to r_0, f can be expressed in terms of r_0. The mass of the particle, m, can also be expressed in terms of r_0 and V. Thus equation (25) can be solved for r_0, and, from this, m can be obtained. Since the volume of a sphere is $(4/3)\pi r_0^3$ and the volume of an elongated ellipsoid of revolution is $(4/3)\pi ba^2$, $r_0^3 = ba^2 = b^3(a/b)^2$. When r_0 and b/a are known, b and a can both be calculated. This completes the analysis in terms of m, a, and b. The algebra involved in the original use of this method differed from that just described, but the underlying principles were the same.

A more recent application of a similar approach has been used to identify the sedimentation coefficients of various intermediates in the polymerization of TMV protein. In this case, m of the protein monomer is accurately known from the amino acid composition, and the dimensions are known from X-ray diffraction analysis of crystals of TMV. The particle can not be well approximated by an ellipsoid of revolution. However, Lauffer and Szent-Györgyi (1955) showed by examination of the appropriate equations that for particles which could not be represented accurately by an ellipsoid of revolution the friction coefficient, f, for the ellipsoid of revolution which could be inscribed within the particle and f for the ellipsoid of revolution which could be circumscribed about the particle were close in magnitude. This permits a reasonably satisfactory approximation of f to be calculated for particles which deviate substantially from the true ellipsoidal shape. By applying these principles, Ansevin and Lauffer (1959) showed that a sedimentation coefficient of 1.9 S corresponded to the monomeric unit. Caspar (1963) extended this line of reasoning to show that cyclical trimers should sediment at approximately 4 S and a double disk, consisting of two rings with 16 monomers each, should sediment at about 20 S. Such calculations permit identification of species observed in ultracentrifugation experiments with particles characterized by other techniques—electron microscopy, for example.

4.3. Differential Centrifugation

Centrifugation is an important tool for the isolation and purification of viruses. It has received extensive use in the years since the pioneering work mentioned in the introduction. Originally, the most widely used technique was differential centrifugation, that is, centrifugation of tissue extracts, bacterial lysates, etc., at low speed, a few thousand rpm, to remove cellular debris, and then at high speed, 20,000–30,000 rpm, to sediment the viruses but leave low-molecular-weight components of the extracts in solution. The process is usually repeated through several cycles. Its effectiveness depends on the fact that most debris, especially if the starting material is of plant origin, has very high sedimentation coefficients, usually many thousand S, while viruses have coefficients of a few hundred S. In contrast, most soluble cellular contaminants have S values of the order of magnitude up to 10 S. Thus the debris sediments at low speeds and leaves the virus and soluble contaminants in the supernatant fluid. In the high-speed centrifugation, the viruses are pelleted, but most of the slower-sedi-

menting material remains in the supernatant fluid. Such sedimenations are frequently carried out in angle centrifuges in which the tubes containing the material are placed in the rotor at an angle. This has the advantage of shortening the path over which sedimentation must take place in order to achieve pelleting. Furthermore, angle centrifuges can be obtained with large-capacity rotors which permit the use of large volumes. The centrifuges can be refrigerated, thereby lessening the likelihood of denaturation of unstable viruses. This technique is well adapted for use when large amounts of tissue extract can be made available, as is frequently the case with viruses which can be propagated in plants, bacteria, tissue cultures, or chicken embryos. The method, however, has one serious limitation. Convection is a problem in angle centrifuges, and this constantly resuspends some of the sedimented material. The result is that separations are not quantitative. This can be a serious problem when only small amounts of virus material are found in the extract. A second potential disadvantage is that any nonviral material with a sedimentation coefficient of a few hundred will not be removed.

4.4. Density Gradient Sedimentation

Sedimentation in a sucrose density gradient circumvents most of the above difficulties, especially that associated with convection. Pickels (1943) employed sucrose density gradients to inhibit convective disturbances in studies on hemocyanin from *Limulus polyphemus*. His method of promoting the gradient was to prepare two hemocyanin solutions of exactly the same composition except that 8–10% sucrose was incorporated in one. The centrifuge tube was half filled with the hemocyanin solution containing sucrose and then completely filled with the other. A few vertical strokes with a glass rod mixed the two fluids partially, "giving a rather uneven but usuable gradient." Stanley (1944) tried this in the purification of influenza virus but decided that is was unnecessary. Brakke (1951), aware of the work of Pickels, extended this procedure by placing a thin layer of a virus solution on top of a centrifuge tube containing a sucrose gradient. To this end he placed sucrose solutions of decreasing concentrations in a centrifuge tube, allowing diffusion to occur for several days. This material was then spun in a centrifuge with a bucket rotor. Potato yellow dwarf virus particles sedimented through this gradient in a zone. This method has since been used widely in the preparation of viruses. It has been refined to the point that proteins and enzymes can be separated (Martin and Ames, 1961). A diagramatic

illustration, taken from a previous volume of *Comprehensive Virology*, of the separation of short and long particles derived from two strains of tobacco rattle virus (TRV) is shown in Fig. 9 (Okada and Ohno, 1976). The use of density gradient centrifugation in the study of plant viruses has been reviewed by Brakke (1960).

The method of density gradient centrifugation has come into such wide use that automated procedures have been developed for establishing gradients. The basic principle involved is that two solutions, usually sucrose solutions, are made up, one with the maximum density desired for the gradient and the other with the minimum density. The tube is filled from the solution of maximum density at the same time that the solution of minimum density is gradually added, with mixing, to the denser solution. Inspection of equation (25) will indicate that sedimentation cannot be at a uniform rate in a density gradient because the density, d, changes with position in the tube, as does the viscosity, η, to which the friction coefficient, f, is proportional. Tables have been published, however, which permit estimation of the sedimentation coefficient from density gradient experiments (McEwen, 1967).

Another way of estimating the sedimentation coefficient from results obtained in sucrose gradients is to compare the sedimentation of the unknown with that of a marker of presumably known S value in the same gradient. If the partial specific volume of the marker is about the

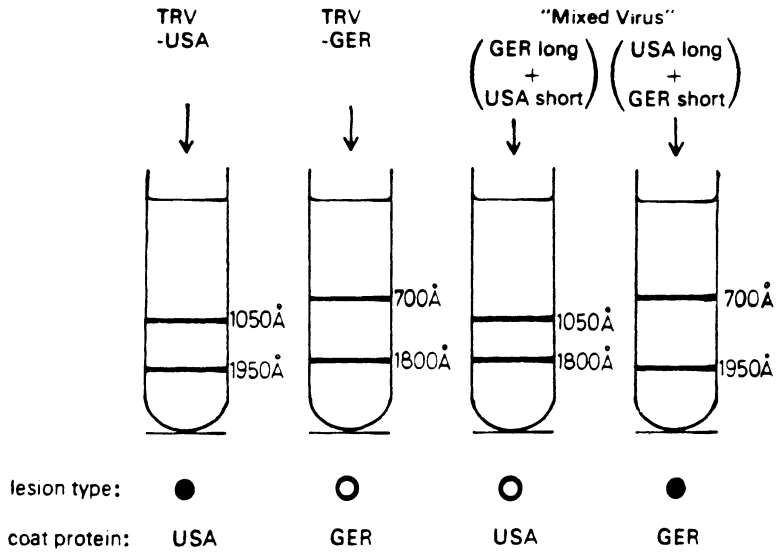


Fig. 9. Sedimentation profiles in a sucrose gradient of two strains and two experimentally mixed TRV systems. Adapted from Okada and Ohno (1976).

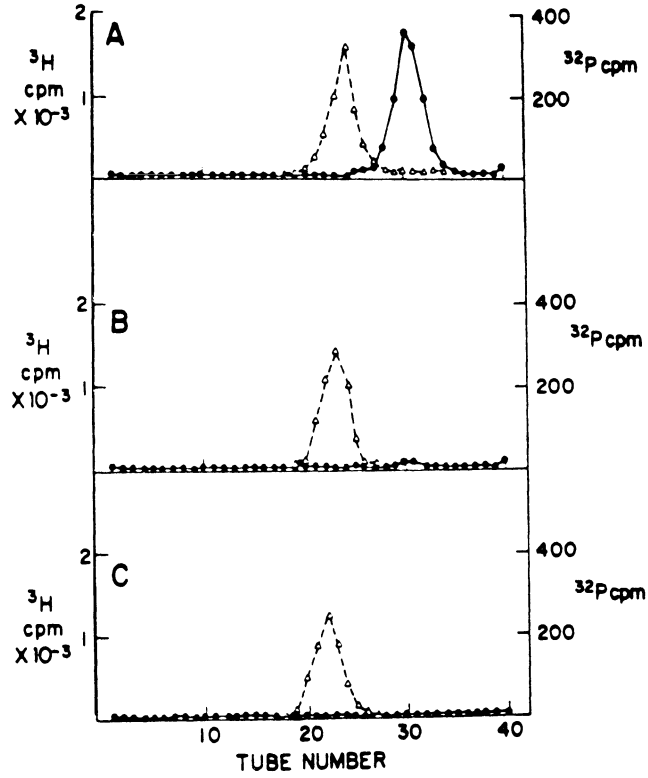


Fig. 10. Sucrose-gradient velocity centrifugation of cellular DNA isolated from purified SV40. SV40 was grown in (A) AGMK cells, (B) CV-1 cells, and (C) BSC-1 cells that were labeled with [^{3}H]thymidine before infection. The virus was purified, broken open with SDS, and sedimented in an SW50 1 rotor for 3 hr at 130,000*g*. Symbols: ●, ^{3}H cpm; Δ, ^{32}P cpm added as a 21 S SV40 DNA sedimentation marker (Levine and Tersky, 1970). Published with the permission of the copyright owner, American Society for Microbiology.

same as that of the unknown, this approach should be valid. In this manner, Gafford *et al.* (1978) estimated the s_w^{20} values of the DNAs from vaccinia, fowlpox, and juncopox viruses, using T4 bacteriophage DNA with an s_w^{20} of 62.1 as a standard.

A very desirable feature of the density gradient method is that small amounts of material can be recovered for bioassay or physical assay. At the end of an experiment, a hole can be punctured in the bottom of the centrifuge tube, and, with the aid of a fraction collector, more rapidly sedimenting materials can be separated from more slowly sedimenting materials. The method can be used to isolate and to separate constituents of viruses. This is illustrated by the results of Levine and Teresky's (1970) separation of cellular DNA from purified SV40 shown in Fig. 10. This result was previously reviewed in

Comprehensive Virology by Aposhian (1975). The method of density gradient sedimentation is ideally suited to problems in which only small amounts of starting material are available. It is also useful in separating materials which differ to only a small extent in sedimentation coefficient. It would be a good method to use for isolating and even separating enzymes in extracts of tissues in which viruses are replicating. The resolving power of the method depends not only on differences in the ratio of m/f of the particles but also on whatever differences might exist in partial specific volumes, V. It also depends critically on the thickness of the layer of extract placed on top of the solution, the thinner the better. Diffusion works against resolution; in this case, diffusion takes place in both directions from the centers of the bands being separated.

5. SEDIMENTATION EQUILIBRIUM

5.1. Theory

When sedimentation is carried out at angular velocities much lower than those used for sedimentation velocity experiments, there is a tendency for solute molecules to sediment toward the periphery and simultaneously to diffuse in the opposite direction. It usually takes a long time for equilibrium to be achieved, but, after it has been achieved, the amount of material sedimenting toward the periphery is exactly equal to the amount diffusing toward the axis at every position in the solution. The amount, dS, sedimenting outward through a cross-section Q in time dt is given by

$$dS = Q\,(dx/dt)\,c\,dt \tag{32}$$

The amount diffusing in the opposite direction is given by equation (10). At equilibrium, these are equal and $(dx/dt)c = D\,(dc/dx)$. When the value of dx/dt from equation (25) is introduced and when, according to the Einstein-Sutherland equation, equation (12), RT/Nf is substituted for D, $\omega^2 xm(1 - Vd)c/f = RT/Nf(dc/dx)$

$$M\omega^2 x\,dx = \frac{RT(dc/c)}{(1 - Vd)} \tag{33}$$

$$M = RT \ln \frac{(c_2/c_1)}{\omega^2(1 - Vd)(x_2^2 - x_1^2)} \tag{34}$$

Since this equation can also be derived from thermodynamics, the method of sedimentation equilibrium is a thermodynamic method for determining molecular weight, M, entitled to the high degree of credibility associated with such methods. For a homogeneous material of molecular weight, M, $\ln c$ is a linear function of x^2. The slope of the line is equal to $M\omega^2(1 - Vd)/RT$. When V, d, ω, and T are known, M can be determined from the slope. This method has been used occasionally to determine M for virus particles, but it is not ideally suited for such purposes because M is normally so large that very small values of ω are required to produce a reasonable concentration distribution from the top to the bottom of the cell. Rotor precession is a problem at low speeds. The method is much better adapted to the study of components of viruses—the coat protein, for example. One difficulty associated with the method is that it requires that c be determined as a function of position from the axis of rotation, x. Many of the usual optical systems found on ultracentrifuges depend on the schlieren principle and yield concentrtion gradients, dc/dx, as a function of x. One way out of this difficulty is to rearrage equation (33) into the following form:

$$\frac{1}{x}\frac{dc}{dx} = \frac{M(1 - Vd)\omega^2 c}{RT} \equiv 2Hc \tag{35}$$

Now dc/dx at various positions, x, can be obtained directly from schlieren patterns. If c_m is defined as the value of c at the meniscus, then c equals $c_m + (c - c_m)$. When this is substituted, the equation becomes

$$\frac{1}{x}\frac{dc}{dx} = 2Hc_m + 2H(c - c_m) \tag{36}$$

The quantity $(c - c_m)$ can be determined readily from the area under the gradient pattern between the value of x chosen and the meniscus. When $(1/x)\,(dc/dx)$ is plotted against $(c - c_m)$, a straight line should be obtained with slope $2H$.

This method of analyzing sedimentation equilibrium data was developed by van Holde and Baldwin (1958). If the material under study is a mixture of substances with different values of M, then this method of treatment will no longer yield a straight line. Thus sedimentation equilibrium is a method for investigating homogeneity.

Sedimentation equilibrium is ideally suited for investigating self-associating systems like tobacco mosaic virus protein. The methods of

analysis required in such cases cannot be described adequately in an elementary discourse such as this. The interested reader is referred to two of the many discussions of this matter (van Holde *et al.*, 1969; Stevens, 1975). As examples, Durham (1972) used this method to study the polymerization of tobacco mosaic virus proteins and Shalaby and Lauffer (1980) studied that of the protein from the flavum strain.

5.2. Density Gradient Sedimentation Equilibrium

One aspect of sedimentation equilibrium is the technique of density gradient sedimentation equilibrium, also called isopycnic centrifugation. In this method, a density gradient is established by centrifugation of a solution containing a heavy salt, such as cesium chloride, until the salt comes to sedimentation equilibrium (Meselson *et al.*, 1957). With cesium chloride, this is feasible because of the low value of the partial specific volume, V. For this reason, as is shown in equation (25), it will have an appreciable sedimentation coefficient, although, because of its small size, it will also have a high diffusion coefficient. At high values of ω, an equilibrium is established in which the concentration of salt, and therefore the density of the solution, varies appreciably with the distance, x, from the center of rotation. If the solution contains nucleic acid or protein in addition to the heavy salt, the protein or nucleic acid will concentrate in a band where the density of the solution is approximately equal to the reciprocal of the effective hydrated specific volume of the macromolecule. To understand this situation, equation (25) must be modified. For m, one must substitute the hydrated mass, that is, the mass of the macromolecule plus any water associated with it, and, for V, the hydrated specific volume, V_h. The reason for this is that the solution under question cannot be treated as a binary system. When these modifications are made, it can be seen from equation (25) that, in those regions of the solution where $V_h d$ is greater than 1, the macromolecule will move toward the axis, while in those regions where $V_h d$ is less than 1, it will move toward the periphery. At the point in the sedimenting column where $V_h d$ is equal to 1, the material will not sediment in either direction, and thus a band will be formed at that point. Detailed theory shows that the band is formed approximately but not precisely at the value of x where d equals $1/V_h$.

In two senses this is a sedimentation equilibrium. The first has already been discussed; the density gradient is established by sedimentation equilibrium involving primarily the dissolved salt. However, the macromolecules concentrated in a band also establish an equilibrium

between diffusion and sedimentation. They tend to diffuse away from the center of the band at the same time that sedimentation tends to concentrate them in the band. The width of the band, therefore, is established by an equilibrium between sedimentation and diffusion of the macromolecule. It is inversely related to the molecular weight: the higher the molecular weight, the sharper the band. The classical illustration of the use of this method is the experiment by Meselson and Stahl (1958) which proved that DNA replication is semiconservative. This experiment was possible because the method is sufficiently sensitive to distinguish between the density of normal DNA and that containing heavy isotopes.

A modification of the isopycnic method is to carry out the sedimentation of the material mixed with cesium chloride in a centrifuge with a bucket-type rotor. After the gradient has been established and the macromolecules have formed one or more bands, fractions can be collected as with sucrose density gradient sedimentation. Figure 11 represents an illustration of the use of this method to separate radioactive pseudovirions of polyoma virus from the virus itself (Qasba *et al.*, 1974). The less dense fraction contains the pseudovirions. The pseudovirion is defined as a viruslike particle with fragments of host nucleic acid inside the protein coat of the virus (Aposhian, 1975).

Proof that the pseudovirions contain host DNA was provided by an experiment in which baby mouse kidney cells were supplied [^{14}C]thymidine before infection or [^{3}H]thymidine after infection with polyoma virus. The viruslike particles produced after infection in both cases were then isolated, purified, and mixed. The results are shown in

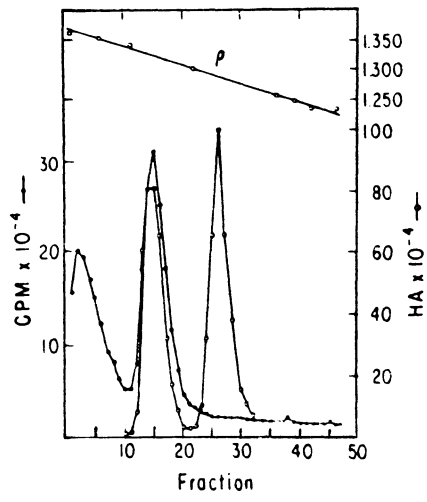


Fig. 11. Equilibrium centrifugation in CsCl of polyoma particles produced in primary mouse embryo cells labeled with [^{3}H]thymidine prior to infection. The spike at lower density on the right represents the pseudovirus and the central spike at higher density, the polyoma virus. From Qasba *et al.* (1974). Reproduced with the permission of the copyright owner, Williams and Wilkins Company.

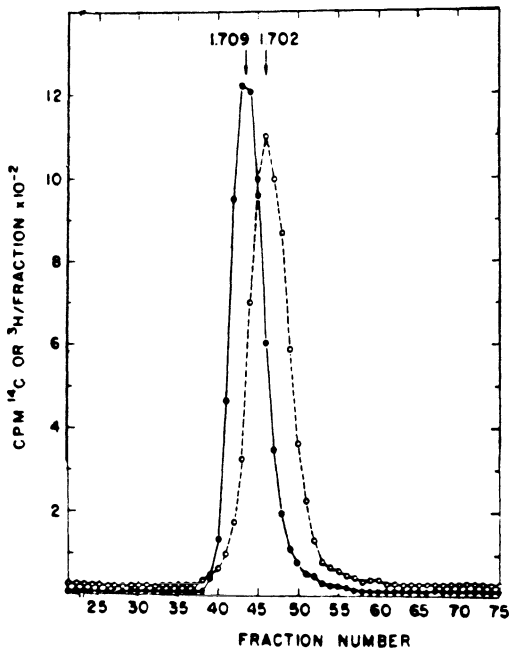


Fig. 12. Buoyant density in CSCl solution of radioactive DNA extracted from particles produced in cells labeled before and after infection. The [^{14}C]-DNA (O---O) was extracted from particles produced in cells whose DNA was labeled with [^{14}C]thymidine prior to infection. The [^{3}H]-DNA (●——●) was extracted from particles produced in cells labeled with [^{3}H]thymidine after infection and was chromatographed on a MAK column to remove components other than the renaturing 20 S component (Winocour, 1968). Published with the permission of the copyright owner, Academic Press, Inc.

Fig. 12. The [^{14}C]-DNA had a density equal to mouse DNA (1.702 g/ml), representing the pseudovirions, and the [^{3}H]-DNA had a density equal to polyoma DNA (1.709 g/ml), (Aposhian, 1975; Winocour, 1968). A more recent illustration of the use of this method was in the isolation of high-density mutants of bacteriophage T5 (Saigo, 1978).

6. CHROMATOGRAPHY

6.1. Countercurrent Distribution

The elementary aspects of the theory of chromatography are closely related to the theory of countercurrent distribution. The countercurrent distribution method depends on the distribution of a solute or solutes between two immiscible solvents. The chemical potential, μ_i, of the ith component of a particular solution, also called the partial molar free energy, is practically equal to the increase in Gibbs free energy of a solution when 1 mol of the ith solute is added to a large volume of solution. The chemical potential of a solute is related to its activity according to

$$\mu_i = \mu_{io} + RT \ln \mathcal{A}_i \tag{37}$$

In this equation, $\mathcal{A}_i$ is the activity of the ith solute and μ_{io} is its chemical potential when its activity is 1. In a different solvent, the chemical potential of the same solute, μ_i', is given by equation (38), where primed quantities refer to solute in this different solvent.

$$\mu_i' = \mu_{io}' + RT\,ln\mathcal{A}_i' \tag{38}$$

If the two solvents are immisicible, the solute will distribute between them when they are shaken together. At equilibrium, the free energy of transfer of solute from one solvent to the other is zero. Therefore,

$$\mu_i = \mu_i' \tag{39}$$

$$\mu_{io}' + RT \ln \mathcal{A}_1' = \mu_{io} + RT \ln \mathcal{A}_i \tag{40}$$

$$RT \ln \mathcal{A}_i'/\mathcal{A}_i = \mu_{io} - \mu_{io}' = \text{constant} \tag{41}$$

At constant temperature,

$$\mathcal{A}_i'/\mathcal{A}_i = \text{constant} \tag{42}$$

When x represents mole fraction, for an ideal solution,

$$x_i'/x_i = \text{constant} \tag{43}$$

For an ideal dilute solution, where c represents concentration in moles per liter or any other measure of mass per unit volume of solution,

$$c_i'/c_i = \text{constant} \equiv K \tag{44}$$

K is the partition coefficient, the ratio of the concentration in the less dense solvent to that in the more dense solvent. This simple expression for the partition coefficient cannot hold for nonideal concentrated solutions, but the most important principles involved in the countercurrent distribution method can be understood by reference to this relationship.

Countercurrent distribution involves distributing a solute or solutes between two immisicble solvents in a large number of sequential separations (Craig, 1950). An apparatus must be available which permits particular samples of a denser solvent to be equilibrated in succession with different samples of a lighter solvent. The idea can be represented diagramatically as in Fig. 13. All of the solute is placed initially into position 0. After equilibration, a fraction, p, is in the upper

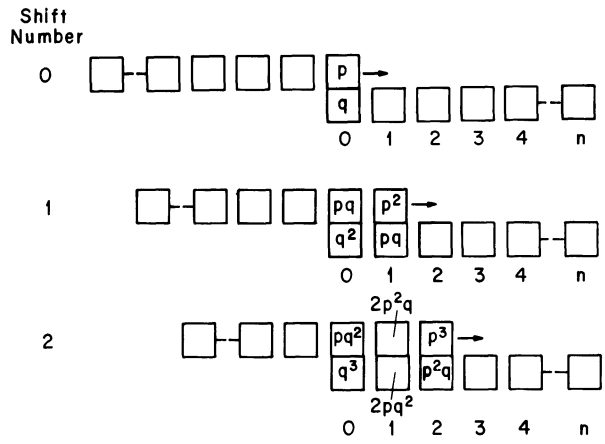


Fig. 13. Diagramatic representation of the countercurrent distribution method. The bottom rack of tubes contains the denser and the top rack the less dense, immiscible solvent. The top rack is moved to the right one place after each equilibration.

solvent and a fraction, q, is in the lower. The sum of p and q is 1, and the ratio of p to q is K. Thus, after 0 shifts, the fraction of material in position 0 is 1, with p above and q below. Now shift the upper set of tubes one place to the right. After this, the total amount of material in position 1 will be p, distributed after equilibration as p^2 above and pq below. The total fraction of material in position 0 will be q distributed after equilibration as pq above and q^2 below. Now make a second shift of the top series of tubes to the right. The total amount of material in position 2 will be p^2, distributed after equilibration as p^3 above and p^2q below. The total amount of material in position 1 will be $2pq$, distributed after equilibrium as $2p^2q$ above and $2pq^2$ below. The total amount of material in position 0 will be q^2, distributed after equilibration as pq^2 above and q^3 below. It should be noted that in all three cases—no shift, one shift, and two shifts—the amount of material in successive positions, taken as the total in the upper and the lower solvents, is given by expanding the binomial, $(q + p)^n$, where n is the number of shifts. By extending this idea, even when n is a very large number, the amount of material in each position will be given by the successive terms of the expanded binomial. p^n will be the total fraction of the material in the upper and the lower solvents combined in the position n, q^n will be the total fraction of the material in position 0, and the fractions in intermediate positions will be given by the successive terms of the expanded binomial. Figure 14 shows the distribution when n is 20 for two different values of K, that is, p/q. The net effect is that the solute is moved to the right. The motivation is the flow of the upper

solvent to the right. The distribution of each solute, however, forms a bell-shaped curve.

The situation can be described as the movement of the upper solvent against a stationary lower solvent. The total distance moved by the upper solvent is proportional to n, the number of separations. The solute, originally at the starting position of the advancing solvent front, will tend to move with the upper solvent, but will be spread out in a broad, symmetrical zone with a peak in the central region. The distance moved by this peak from its starting position will be less than the distance moved by the solvent front. The ratio is called R_f. The value of R_f is high for systems for which the value of K of equation (41) is high. It is obvious from Fig. 14 that solutes with different values of K and, therefore, of R_f can be separated by the method of countercurrent distribution.

Countercurrent distribution is of great historical significance in virus research. The coat protein of tobacco mosaic virus was the first for which the complete amino acid sequence was determined (Tsugita *et al.*, 1960; Anderer *et al.*, 1965). Tryptic digests of the protein were separated into fractions by countercurrent distribution between 2-butanol and 0.1 M dichloroacetic acid (Gish *et al.*, 1958). However, the method has been largely superseded by less cumbersome procedures and is therefore largely of historical and theoretical importance in virus research.

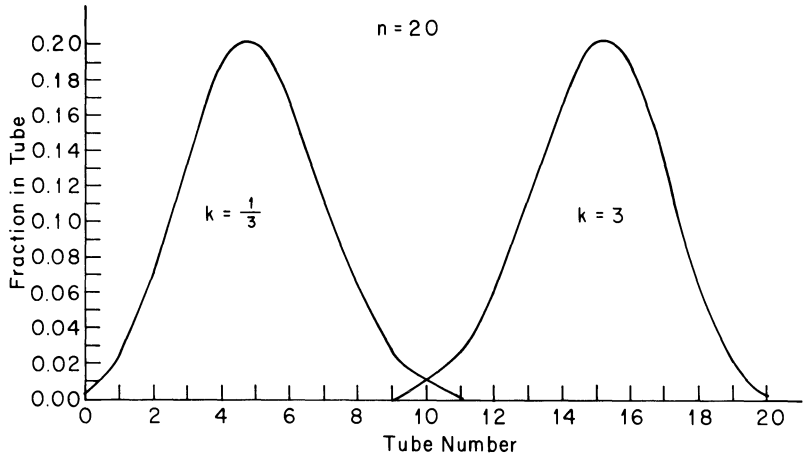


Fig. 14. Theoretical distribution of two ideal solutes after 20 separations in the countercurrent apparatus. The curve to the right is calculated for a solute with K value of 3 and the one on the left for a solute with a K value of ⅓ in the two solvents.

6.2. Column Chromatography

Column chromatography is in some ways the analogue of countercurrent distribution. In this case, solvent is moved past a stationary solid which has the ability to combine with or adsorb solute. Thus column chromatography depends on a distribution of solute between a solid material and a moving solvent. A more extensive discussion is presented by Haschemeyer and Haschemeyer (1973). Even at the theoretical level, there are some points of similarity between countercurrent distribution and column chromatography.

An oversimplification of the theory of column chromatography, presented solely for the purpose of illustrating basic principles, can be based on the assumption that the solid contains binding sites or adsorption sites for the solute molecules with uniform affinity for solute, independent of the extent of binding. Represent the solute by S and the binding sites by B and let there be n moles of binding site on all of the solid phase in contact with 1 liter of solvent. The binding can then be represented by equation (45) and the mass action equation is formulated as equation (46):

$$S + B = SB \tag{45}$$

$$[SB]/[S]\,[B] = K_{eq} \tag{46}$$

The concentration of free binding sites, $[B]$, is n minus the concentration of bound sites, $(n - [SB])$. When the fraction of sites combined is small, equation (47) results.

$$\frac{[SB]}{[S]} = nK_{eq} \equiv K' \tag{47}$$

In equation (47), K' is a distribution coefficient between the solid and the solvent. Equation (47) is completely analogous to equation (44) for the distribution of solute between two immiscible solvents. In real situations, the distribution of solute between the solid phase and the solvent is much more complicated than indicated by this simple derivation. However, the principle remains valid that column chromatography can be understood in terms of an equilibrium between solute molecules adsorbed onto or combined with the solid and solute molecules in solution.

If a column is packed with an adsorbant in heavy suspension in a solvent and if a thin layer of a solution containing material capable of

being adsorbed on the solid is placed on top of the column, then that solute will distribute itself between the adsorbant at the top of the column and the solvent in contact. If the column is now "developed" by the device of moving fresh solvent down through it, the band of adsorbed solid in equilibrium with adjacent solvent will move down the column. The situation is analogous to that observed in the countercurrent distribution except that, instead of having n discrete advances of solvent, there is continuous flow, which can be considered to be an infinite number of infinitely small advances. If the theory, expressed as equation (47) for the grossly oversimplified conditions postulated, actually applied in the real case, the analogy with countercurrent distribution would be perfect. Even in the real situation, however, the zone of adsorbed material does migrate, it does tend to broaden out, and it does have a value of R_f which depends in general on how strongly the solute is adsorbed to the solid. Substances with significantly different values of R_f can be separated. The resolution depends on many factors; obvious among them are the values of R_f of the various solutes in the particular solvent–adsorbant system, the length of the column, and the thinness of the layer of solution initially applied.

Many different kinds of solid can be used to pack columns. Some, like charcoal, adsorb solutes by nonpolar interactions, and others, like alumina, silica gel, and calcium phosphate gel, adsorb by polar interactions. Hydroxyapatite, $Ca_{10}(PO_4)_6(OH)_2$, is frequently used. Other solids are ion-exchange resins. Some, like Dowex-50, are salts of a resin containing SO_3^- groups. This can bind positively charged molecules by displacing the cations origianlly on the resins. Other cation exchangers include carboxymethyl cellulose (CM-cellulose), phosphocellulose, and cellulose containing a sulfoethyl group (SE-cellulose). There are also anion exchangers, resins, or gels with positive groups attached. The diethylaminoethyl group is frequently used, as in DEAE-cellulose. In addition, solids with aminoethyl, triethylaminoethyl, and guanidoethyl groups are attached to solids to form anion exchangers. Not only cellulose but cross-linked dextran (sephadex) and polyacrylamide gel can be used. Ion exchangers are frequently used for separating proteins and amino acids.

In order for a band of solute to move along the column as it is developed, the value of the distribution coefficient between solid and solvent must be in an intermediate range where appreciable amounts of solute are found in both the solid and the liquid phase at equilibrium. If the binding is very weak, practically all of the solute will remain in solution; if it is very strong, so little will be in solution that, for all practical purposes, the solute does not move as the column is developed

with solvent. This is a frequently encountered situation. For example, if a mixture of proteins is placed on a DEAE-cellulose column, most or all will be bound strongly at high pH where most proteins have high negative charges. If, however, such a column is eluted with a gradient of pH, beginning with high pH and ending with low pH, one by one the various proteins in the mixture will become soluble because the negative charge will be reduced sufficiently to reduce the binding or to destroy it altogether. Thus one by one the proteins will be released and moved down the column as the gradient passes through. Another method of eluting is with a salt gradient. This can be thought of either as reducing the ionic attraction between the charges attached to the solid and those on the solute in a way understood for simple ions in terms of Debye-Hückel theory, or as simple mass action. In either case, the less strongly bound solutes will be released in low salt concentrations and the more firmly bound ones in higher concentrations.

In some cases, a ligand which binds selectively to some proteins but not to others can be attached covalently to some matrix material. A column made of such material will then retard only those proteins which bind to the ligand. If several proteins bind to the same ligand, the extent of retardation depends on the relative affinities of the ligand for the proteins. Materials which do not bind with the ligand pass through as soon as the exclusion volume has passed. When the binding between a protein and a ligand is very strong, elution can be effected by changing pH, temperature, ionic strength, etc., or by adding an even more strongly binding ligand to the elution solution. Sometimes cleavage of the matrix–ligand bond is necessary. The ideal ligand for isolating a specific protein is the antibody to that protein.

An interesting column combining several of the features just discussed is the MAK column used for DNA purification. It consists of serum albumin rendered basic by methylation, supported by kieselguhr (or celite). Being strongly basic, it binds DNA. Elution is effected with a salt gradient (Mandell and Hershey, 1960). This column was used in the purification of the DNA from pseudovirions and polyoma virus (see Fig. 12).

Many ways exist for recovering separated solutes from the columns. When this method first came to prominence, it was used extensively for the separation of pigmented materials; hence the name "chromatography." The various colored bands were clearly visible on the columns. They could be recovered simply by cutting slices containing the separated components and extracting with solvent. Another method of recovering a solute is to continue development until the band of that material goes beyond the end of the column. It can be collected

in a fraction collector. As development continues, other bands will come off later and will be found in other fractions.

Column chromatography can be used to purify virus preparations. LoGrippo (1951) showed that poliomyelitis and Theiler's viruses could be adsorbed onto and eluted from the basic resin, Amberlite Xe-67, and Shainoff and Lauffer (1956) used this same resin to remove the pigments from extracts of plants infected with southern bean mosaic virus. The separation was complete.

An interesting hybrid procedure related to both column chromatography and countercurrent distribution, called partition chromatography, is one in which a column is packed with highly hydrated silica gel, starch gel, or cellulose gel. The colum is developed with a nonpolar solvent. The solute can be thought of as being partitioned between the water held in the gel and the nonpolar solvent, in this sense resembling the countercurrent distribution method. This technique was introduced by Martin and Synge (1941).

6.3. Paper Chromatography

Paper chromatography is widely used in biochemistry, especially for the separation of amino acids. In the simplest examples of this technique, a spot or streak of a solution containing the substances to be separated is applied near the bottom of a strip of filter paper. The bottom of the strip is then dipped into a nonpolar solvent, which slowly rises through the paper by capillary action. Chromatographic separation of solutes takes place, those with the greater affinity for the nonpolar solvent moving closer to the advancing edge of the solvent. Remarkable separation can be achieved by developing in two directions. A spot in the mixture to be separated is placed near one corner of a square sheet of paper and developed with a nonpolar solvent. It is then dried and developed at right angles, usually with a different nonpolar solvent. It is possible to separate a protein digest into its components by this procedure.

6.4. Thin-Layer Chromatography

Thin-layer chromatography (TLC) is a technique rather similar to paper chromatography. A thick slurry of some supporting medium such as silica gel, alumina gel, powdered cellulose, etc., is spread onto a flat plate in a uniform thin layer. After the water has evaporated,

chromatography can be carried out as with paper. Two-dimensional thin-layer chromatography is used like two-dimensional paper chromatography to separate amino acids from protein digest. The resolution is better than with paper.

6.5. Molecular Exclusion Chromatography

Molecular exclusion chromatography (MEC), also known as gel filtration or molecular sieve chromatography, is a method for separating solutes on the basis of molecular weight. It is extensively used for separation of proteins. The essence of the method is to pack a column or coat a plate for TLC with a slurry made from dehydrated beads or particles which swell in solvent to produce gels with more or less uniform pore sizes. The packed column or thin layer will then contain solvent outside the swollen gel particles, which constitute the excluded volume, and solvent inside the swollen beads of gel. Various materials such as agarose, dextran and polyacrylamide (Sephadex and Bio-Gel) are available with different ranges of pore size. When a solution of proteins, for example, is placed on the column or on the thin layer, those proteins and contaminating materials or breakdown products small enough to pass through the pores in the gel go into the interior of the gel particles and are thereby retarded. Proteins and other materials of such large size that they cannot penetrate are retained in the excluded volume outside the gel particles. On development, such particles pass through with the solvent and come off the column as soon as the exclusion volume has passed through. Molecules of intermediate size pass through at intermediate rates. It is possible to separate peptides or proteins differing in molecular weight by a factor of 2 or sometimes even less.

7. ELECTROPHORESIS

7.1. Theory

"Electrophoresis" is the name given the movement of charged macromolecules in an electronic field. The simplest possible case is represented by a spherical macromolecule in a solution containing no ions other than those obtained by the ionization of the spheres themselves. For such a system, the electrical force on the sphere is the product of its net charge, Q, and the strength of the electric field, E,

expressed as the potential drop in volts per centimeter. This electrical force causes the sphere to migrate toward the electrode of opposite sign. As the sphere migrates, it encounters a force of friction equal, according to equation (13), to $6\pi r\eta v$, where v is the velocity and r the radius of the particle. These two forces are equal; thus equation (48) results.

$$QE = 6\pi r\eta v \tag{48}$$

$$u \equiv v/E = Q/6\pi r\eta \tag{49}$$

In equation (49), u is the electrophoretic mobility, the velocity per unit of field. The potential at the surface of such an isolated sphere, ζ, is known from elementary physics to be given by equation (50), in which D is the dielectric constant.

$$\zeta = Q/Dr \tag{50}$$

When equations (49) and (50) are combined, equation (51) results.

$$u = D\zeta/6\pi\eta \tag{51}$$

This equation holds only for charged spheres in solutions in which small ions are at extremely low concentrations. When the macromolecule is in an electrolyte solution such as a buffer or a neutral salt with molar concentrations in the neighborhood of 0.01–0.10, equation (51) no longer holds. The electrolyte does two things, both by forming a diffuse layer of oppositely charged ions about the spherical particle. One is to reduce drastically the potential at the surface of the sphere per unit of net charge, and the other is to affect the hydrodynamic interaction between the sphere and the solution. Both of these effects are taken into account at the next level of electrophoretic theory and result in equation (52), which is valid for large spheres in solutions of sufficient ionic strength to give large values of the Debye–Hückel constant, κ.

$$u = D\zeta/4\pi\eta \tag{52}$$

A derivation of equation (52), in which somewhat different symbols were used, was presented by Mueller (1943). A general equation encompassing the extreme situations represented by equations (51) and (52),

TABLE 3

Selected Values of the Debye–Hückel Constant

μ	κ(water at 25°C)
0.001	1.036×10^6
0.005	2.35×10^6
0.02	4.63×10^6
0.10	10.37×10^6
0.20	15.48×10^6

in addition to intermediates, was presented by Henry (Mueller, 1943):

$$u = \frac{D\zeta}{6\pi\eta} f(\kappa r) \tag{53}$$

From Debye–Hückel theory, it is known that κ is proportional to the square root of ionic strength, μ. When water is the solvent, at 25°C,

$$\kappa = 0.327 \times 10^8 \sqrt{\mu} \tag{54}$$

where μ is the sum of the concentration of each ion multiplied by the square of its valence, all divided by 2. Selected numerical values of κ for aqueous solutions at 25°C are shown in Table 3. In equation (53), $f(\kappa r)$ should be read "function of κr." Table 4 presents that function for a few selected values of κr. It can be seen, therefore, that when κr is very small, as it is in extremely dilute solutions of electrolyte, especially when r is very small, equation (53) reduces to equation (51). It is also evident that when both κ and r are large, equation (53) reduces to equation (52).

The Debye–Hückel theory yields an expression for the relationship between ζ and Q for spherical particles, as shown in

$$\zeta = \frac{Q}{Dr(\kappa r + 1)} \tag{55}$$

Here again, it can be seen that when κr is much smaller than 1, equation (55) reduces to equation (50). When Q is replaced by $4\pi r^2\sigma$, where σ is the net surface charge density, equation (56) results.

$$\zeta = \frac{4\pi\sigma(1/\kappa)}{D(1 + 1/\kappa r)} \tag{56}$$

TABLE 4

Selected Values of $f(\kappa r)$ in the Henry Equation

κr	$f(\kappa r)$
0	1.000
1	1.034
10	1.250
100	1.463
∞	1.500

When this is substituted into equation (53),

$$u = \frac{4\sigma(1/\kappa)f(\kappa r)}{6\eta(1 + 1/\kappa r)} \tag{57}$$

Even at this level, the theory of electrophoresis is only approximate. More accurate solutions of the key equations are presented in tabular form, for example, in Bier (1959). Equation (57) shows how electrophoretic mobility can be manipulated. Such manipulation is useful when one wishes to use this method to separate proteins. Mobility is inversely proportional to viscosity, but changing the viscosity of the medium would affect the mobilities of all macromolecules by the same fraction; it therefore can do nothing to change the resolution in separation methods. Mobility is a function of κ and, therefore, of ionic strength. For spheres of large radius, where κr is much greater than 1, the effect of changing κ is proportionately the same for all spheres. However, the effect of changing κ on small spheres is quite different from changing κ for large spheres. Therefore, altering κ by changing ionic strength can alter the resolution of large from small spheres. Overwhelmingly the most important contribution to mobility is σ, the surface density of net charge. This is highly sensitive to pH and differs widely from macromolecule to macromolecule, because the ionizable groups on the surface differ widely. Thus adjustment of pH is the most effective means of changing the relative mobilities of macromolecules in a single solution. When ions are adsorbed, they change σ. Since the binding of ions by various proteins is an individual property of the proteins, some alteration in resolving power can result from specific ion binding, which is a function of ionic strength and ionic composition.

An interesting minor consideration is that it is possible to change net surface charge density without changing the net charge per unit

mass. If protein molecules in solution aggregate or polymerize without changing ionization, the net charge per unit mass remains unchanged. However, the surface area per unit mass decreases. Thus the net surface charge density increases, and one should expect the electrophoretic mobility to increase. An interesting example of this is afforded by the electrophoretic studies of Kramer and Wittmann (1958) on tobacco mosaic virus protein. In 0.075 ionic strength buffer at pH values near 6, both polymerized and unpolymerized proteins coexist. The negative mobility of the polymerized protein is nearly three times that of the unpolymerized protein even though hydrogen ions are bound during polymerization, thereby actually reducing the net negative charge per unit mass. The explanation is that the decrease in surface area per unit mass on polymerization greatly overbalances the decrease in net charge per unit mass, resulting in a big increase in surface charge density.

7.2. Measurement of Field Strength

Electrophoretic mobility is defined as the electrophoretic velocity divided by the electric field strength. The velocity is simply the distance moved divided by the time. The field strength is the potential drop divided by the distance between the two points at which potential is measured—in other words, potential drop per unit length. It is usually difficult to measure directly the potential drop in the region where electrophoresis is being observed. For that reason, an indirect method, based on Ohm's law, is used. Ohm's law can be expressed in terms of

$$V = RI \tag{58}$$

In this equation, V is potential drop in volts, R is resistance in ohms, and I is current in amperes.

$$E = V/l = RI/l \tag{59}$$

In this equation, l is the distance over which the potential drops, but R/l in a solution is $1/KA$, where K is the specific conductance of the solution and A is the area of cross-section at the point of observation. When this is substituted into equation (59), equation (60) results.

$$E = I/KA \tag{60}$$

Thus the field strength at the point where electrophoresis is taking place is determined by measuring the current, I, flowing through the entire

assembly, and by dividing it by the product of the specific conductance of the medium, K, and the area of cross-section, A. Specific conductance is determined in an independent experiment using a conductivity cell and a Wheatstone bridge assembly. The area of cross-section at the point of observation, A, must be determined beforehand. If electrophoresis is being observed in some sort of cell, a convenient method of determining A is to fill the cell with mercury, determine the weight of mercury, and, from this and the density of mercury, determine the volume of the cell. It is usually possible to measure the length of the cell with some precision. Therefore, A can be calculated.

7.3. Moving Boundary Method

Various methods of studying electrophoresis are described in Bier (1959). The method of choice for making absolute measurements of electrophoretic mobility is the moving boundary technique of Tiselius. A boundary is established between a solution of a macromolecule or macromolecules in an appropriate buffer and its dialysate in a U-shaped cell. The cell is then connected to a reversible electrode system and electrophoresis is carried out. Each macromolecular component of the solution migrates at its own rate and establishes a concentration boundary between regions where it is present and absent. This concentration boundary can be observed by optical means, the most frequently used of which is the schlieren method. Since this system detects concentration gradients, the boundary shows up as a peak or spike. The distance the spike moves in unit time divided by the magnitude of the electric field provides a measure of the electrophoretic mobility of the particular component involved.

An outstanding example of the use of moving boundary electrophoresis to study the electrophoretic mobility of viruses and components of viruses can be found in the work of Kramer and Wittmann (1958) on tobacco mosaic virus and its protein. Figure 15 shows the mobilities of coat proteins from three strains of tobacco mosaic virus, as well as the mobilities of the viruses themselves taken from an earlier publication. Perhaps the most instructive feature of these results is the identity of the mobility of each of the three strains of the virus with that of its polymerized protein in the pH range between the isoelectric point and approximately pH 6. This means, according to equation (57), that the surface charge densities of the polymerized protein and of the virus are identical over a wide range of pH for all three strains. There is one inescapable conclusion. Since mobility is propor-

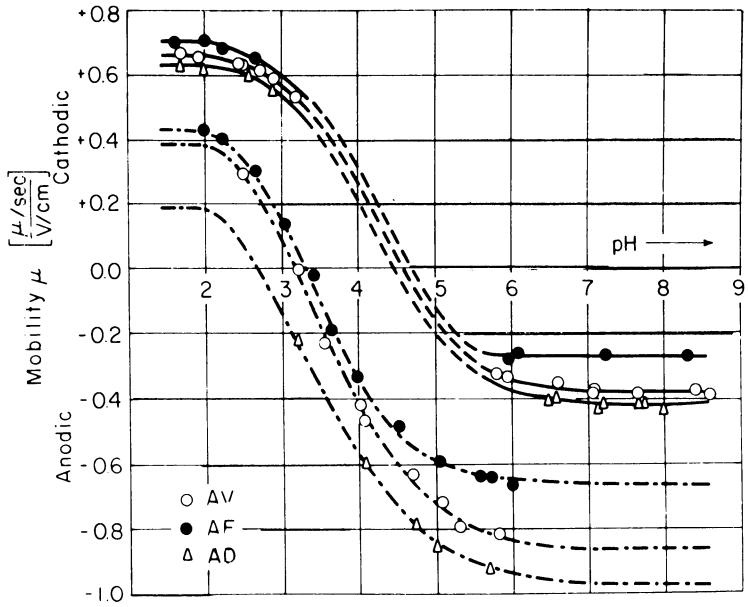


Fig. 15. Electrophoretic mobility of the protein from three strains of TMV. AV, vulgare; AF, flavum; AD, Dahlemense. From Kramer and Wittmann (1958). Reprinted by permission of the copyright owner, *Zeitschrift für Naturforschung*.

tional to net surface charge density, since charge must be conserved, and since RNA is rich in negatively ionizing groups, particularly at the higher pH values, positive ions must be bound when the viral proteins coat their RNAs. An experiment carried out by Stevenson and Lauffer (Lauffer, 1975) showed that, during reconstitution of TMV from protein and RNA in an unbuffered system, the pH increased from pH 6.5 to 7.8, showing that hydrogen ions are bound. A much smaller pH change was associated with protein polymerization initially at pH 6.5. This experiment tends to confirm the deduction from the electrophoretic mobility experiments.

One of the most reliable aspects of electrophoretic mobility theory is that, other things being held constant, mobility is directly proportional to net charge. It is instructive, therefore, to compare the change in charge as determined by electrophoretic mobility experiments with the change indicated by hydrogen ion titration. Since four hydrogen ions per protein monomer are dissociated between the isoionic point of the coat protein of the common strain of tobacco mosaic virus protein and pH 8, and since no other ions are bound in this region, a fixed point in the calculation is that the charge on the protein is -4 per monomer at pH 8. With this result, it is possible to calculate the charge per

monomer for the unpolymerized protein at pH values between about 6 and 8.5 from the ratio of the electrophoretic mobility at a particular pH to that at pH 8. The results are shown in comparison with the charge determined from hydrogen ion titration over the same range in Fig. 16. The agreement is very good for unpolymerized protein. However, at around pH 6, the protein polymerizes. It binds hydrogen ion during polymerization. Titration studies show that, at pH 5.5, polymerized TMV protein has a charge of -0.92 per monomeric unit. At this pH value, there is also no binding of other ions, as far as can be detected. With this value fixed, the net charge at other pH values for polymerized protein can be determined from the ratio of electrophoretic mobility at particular pH values to that at pH 5.5. The results are also displayed in Fig. 16. In contrast to the result obtained with unpolymerized protein, the charge thus determined from electrophoretic mobility is greatly different below pH 5.5 from that determined by hydrogen ion titration of polymerized protein. For example, at pH 3.2 the charge from mobility is zero, but that from titration is +4.4. At this pH value both K^+ and Cl^- ions are bound, but the excess of Cl^- over K^+ bound is 4.2 per monomer. It is thus apparent that the electrophoretic mobility of

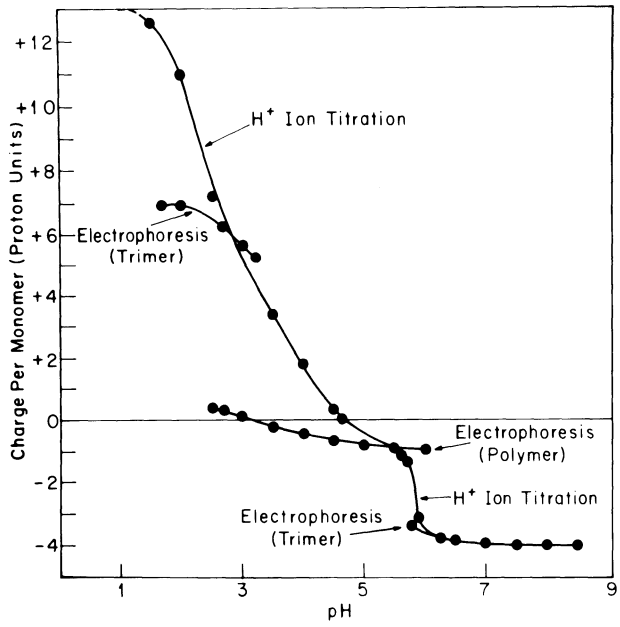


Fig. 16. Charge on TMV protein at 4°C from H^+ ion titration or electrophoresis. From Lauffer (1978). Reprinted with the permission of the copyright owner, Elsevier North-Holland.

polymerized TMV protein is strongly affected by the binding of ions other than H^+ (Lauffer, 1978; Shalby *et al.*, 1968; Banerjee and Lauffer, 1971).

Moving boundary electrophoresis has been used extensively to resolve protein solutions into their components. Each component establishes a boundary in both the ascending and descending arms of the U-shaped tube which shows up in the schlieren optical system as a spike. One of the conspicuous successes of the method was the resolution of blood plasma into several components. The resolving power of this method obviously depends on the difference between the mobilities of various components. Each spike corresponds to at least one component, but if several have similar mobilities, a single spike will include all. Other methods of separation resolve blood plasma into more components.

The Tiselius electrophoresis apparatus can be used to purify proteins and viruses. The most rapidly moving component of a multicomponent system can be obtained in pure solution and can be isolated from the rest. The same is true for the most slowly migratng component. An obvious limitation is that considerable material will be wasted in regions where more than one component remains. This method has been used to purify southern bean mosaic virus (Lauffer and Price, 1947).

7.4. Electrophoresis in Support Media

7.4.1. General Considerations

Over the past two decades, the use of the method of moving boundary electrophoresis has declined greatly. Basically, it is a tedious procedure. It is still the standard method for actually measuring electrophoretic mobilities. However, for the purpose of resolving complex biological extracts into their components, it has the major drawback of requiring significant amounts of material. Other methods of electrophoretic separation have been developed which require only small amounts. These involve the movement through support media like filter paper or gels. A further advantage of such methods is that they require relatively simple experimental arrangements.

A major disadvantage of electrophoresis on support media is that it is impossible to obtain absolute values of mobility. One reason is that the support medium obstructs the movement of the macromolecules; they must follow an irregular path around the stationary obstructing

material. All that one can observe is net movement. There is an additional problem, perhaps even more serious. Whenever the surface of the support medium takes on an electric charge, either by adsorption of ions or by ionization, then, when an electric field is applied, liquid will move past the solid by electroosmosis. The liquid will have a charge opposite that of the surface and will move toward the electrode with charge opposite that of the liquid. Such movement of the solvent can alter considerably the net movement of macromolecular solute.

7.4.2. Paper Electrophoresis

Electrophoresis on paper is widely used. A streak of a solution containing a mixture of materials to be separated is made on a piece of filter paper saturated with buffer. The ends of the paper are dipped in buffer solutions connected to electrodes. Provision is made to minimize evaporation of solvent. When an electric current is passed through the system, the components of the material in the original streak separate into zones which migrate distances depending in part on the electrophoretic mobilities of the components. At the end of the experiment, the position of each component can be located by staining or by some other method. If desired, the paper can be cut and the material at given regions can be eluted and analyzed. The method has good resolution, but the separation of zones is somewhat impaired by diffusion in both directions. An alternative way of analyzing the results of paper electrophoresis is to measure with a densitometer the intensity of the stain after treatment with an appropriate dye. A spike will be obtained for each zone, and the area under each spike should be proportional to the total amount of protein in the zone. Patterns can be obtained which are rather similar in appearance to the schlieren patterns obtained with moving boundary electrophoresis. There is a major difficulty associated with filter paper electrophoresis; in some cases, the paper adsorbs protein so that the zones leave smears behind when they migrate. Cellulose acetate sometimes give better results. It is possible to carry out large-scale operations for purification purposes by packing a column with cellulose fibers and then carrying out zone electrophoresis through the column.

A two-dimensional combination of paper electrophoresis and paper chromatography is used frequently to map or "fingerprint" tryptic digests of proteins. Chu and Francki (1979) used this method on tryptic digests of the coat protein of tobacco ringspot virus. The digests were first subjected to electrophoresis on paper followed by chromatography

in *n*-butanol–pyridine–acetic acid–water at right angles. The peptides were detected by spraying with ninhydrin. Another recent example of the use of this approach is the analysis of the tryptic digests of the proteins from fowl plague virus and virus N (Harms *et al.*, 1978). In this instance, precoated cellulose plates were used and the peptides were detected by autoradiography.

7.4.3. Molecular Exclusion Electrophoresis

The resolution obtained with paper electrophoresis is not subtantially greater than that obtained by moving boundary electrophoresis for reasons similar to those which limit the former method. However, a method has been developed which combines molecular exclusion effects with electrophoresis, a method which resolves macromolecules on the basis of both charge and molecular weight. Electrophoresis of blood plasma in a gel formed from partially hydrolyzed starch resolves the serum into many more components than does either moving boundary electrophoresis or paper electrophoresis. At least partial understanding of this can be achieved by referring to the previous discussion of diffusion in gels. The equations relating the difffusion coefficient measured in a gel to that obtained in free diffusion are based in part on equations for the obstructing effect of the network of swollen fibers of the gel substances on the movement of solute through the liquid within the gel. This obstructing effect is equal to $(1 - \phi)/[1 + (\alpha - 1)\phi]$, where α has a value of 5/3 for randomly oriented threads or rods. Thus the effect reduces to $(1 - \phi)/[1 + (2/3)\phi]$. The symbol, ϕ, was originally defined as the volume fraction occupied by the swollen fibers of the gel substance. However, this is too simple a definition; ϕ is really the volume fraction from which macromolecular solutes are excluded. The center of a spherical macromolecule can get no closer to the axis of a swollen cylinder than a distance equal to the sum of the radii of the swollen cylinder and of the macromolecule. The macromolecule is therefore excluded from a volume equal to that of a cylinder, with the length of the fiber and a radius equal to the sum of the radii of the swollen fiber and of the macromolecule. The larger the macromolecule, the greater is this excluded volume. When the macromolecule is nonspherical, for example, a rod, then the excluded volume is a complicated function of the dimensions of the macromolecule, depending heavily on length. From all of this, it is clear that the excluded volume within a gel differs for different macromolecules passing through that gel, depending on the size and shape of the macromolecule. ϕ varies the same way and, therefore, so

does the retarding effect. This same retarding effect operates when particles move as a result of a potential gradient in electrophoresis as it does when particles move under the influence of a concentration gradient in diffusion. The result is that the retarding effect of the gel depends on the size and shape of the macromolecule. The motivating force on each macromolecule still depends on its net surface charge density. Thus migration through a gel in an electric field can separate particles simultaneously on the basis of surface charge density and molecular size and shape.

Polyacrylamide gel is now used extensively. Many different modifications of this technique are available. One is to pour a thin sheet of gel on a plane surface. A trough can be cut into the gel to accommodate the sample. It is then connected to electrodes for electrophoresis. One modification is first to separate components on the basis of charge density by electrophoresis on a paper strip. Then the strip can be put in the slot and the electrophoresis can be performed in the retarding gel at right angles to the original separation. In this manner, samples of blood serum have been resolved into more than twenty components, consisting of groups the members of which have nearly identical mobilities but different molecular weights. An example of the use of two-dimensional polyacrylamide gel electrophoresis to separate tryptic digests of picornavirus gene products is illustrated in Fig. 17. Another example involves a study of the tRNAs of various mutants of the bacteriophage, BF23 (Ikemura *et al.*, 1878). In this study, ^{32}P-labeled RNA was first subjected to electrophoresis in one dimension in a 10% polyacrylamide gel. A strip containing the 4–6 S RNAs was cut and applied to a 20% gel for electrophoresis at a right angle. Resolved components were detected by autoradiography. In this way, it was shown that the loss of phage suppressor activity in mutants was always accompanied by the loss of certain tRNAs.

The obstructing effect, dependent on ϕ, obviously varies with the concentration of the gel. Other things being equal, the more concentrated the gel, the higher the value of ϕ and therefore of the retarding effect. Indeed, gels can be made so concentrated or so highly cross-linked that they actually act as filters for the largest macromolecules.

7.4.4. Disc Electrophoresis

An interesting modification is called discontinuous or disc electrophoresis. The major portion of the tube is filled with a highly retarding gel, called the running gel. Above it is placed a less retarding gel,

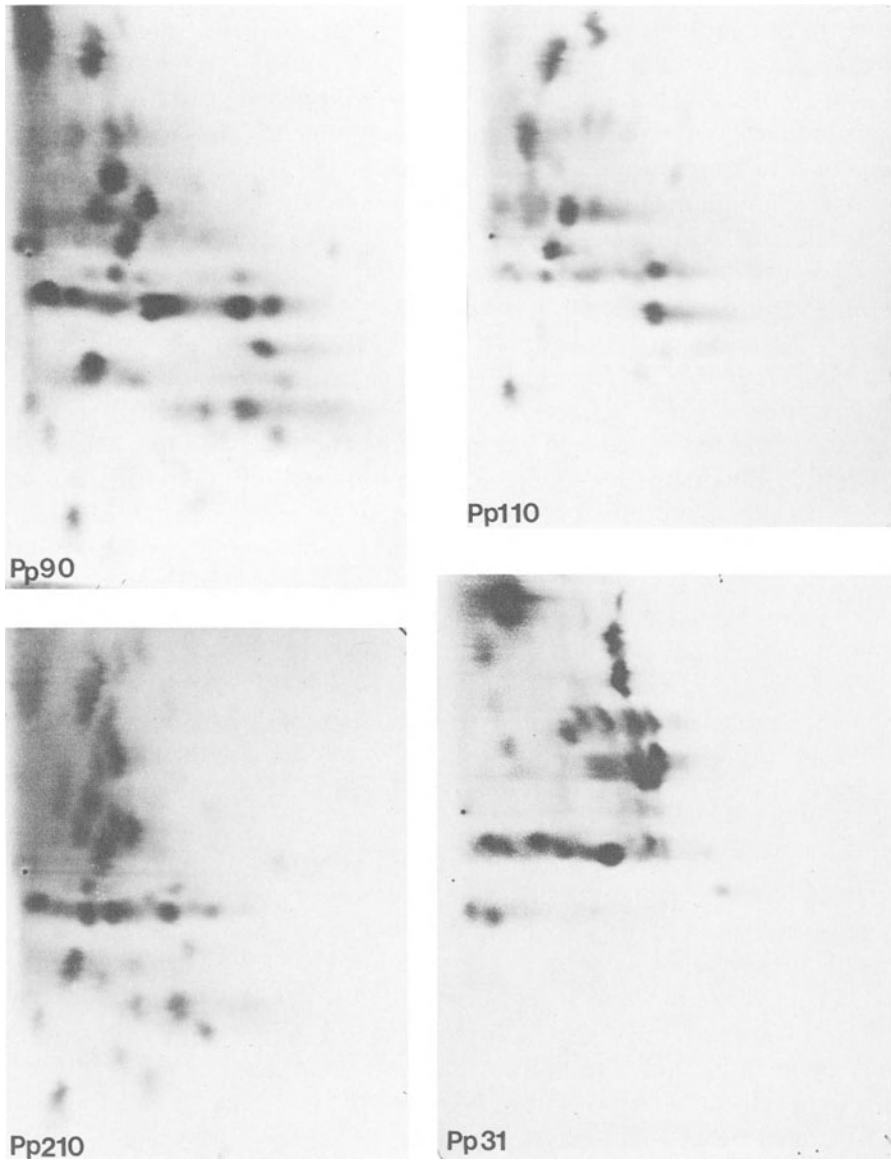


Fig. 17. Relation between the primary polyprotein of poliovirus (Pp210) and virion proteins (VP2, VP1, VP3), some cleavage intermediates, and the anomalous product Pp31, as indicated by tryptic peptide autoradiographs of bands isolated from polyacrylamide gels. From Abraham and Cooper (1975). Published with the permission of the copyright owner, Cambridge University Press.

called the spacer gel, and above that the sample, sometimes in a thin layer of gel. The buffer—which consists of an amine and its hydrochloride—is around pH 8 in the sample and spacer, but around 9.5 in the running gel. The electrode vessel contains glycinate ions instead of chloride. In sample and spacer at pH 8, glycinate has a lower mobility than proteins and a much lower mobility than chloride. A sharp boundary results between chloride and glycinate, with the protein concentrated between them. When the boundary moves into the running gel at pH 9.5, glycinate moves faster than proteins, so the two are separated in a uniform medium according to their individual mobilities (Laemmli, 1970). An example of the separation of viral polypeptides by this method is shown in Fig. 18. Hutchison *et al.* (1967) used polyacrylamide gel disc electrophoresis to separate ϕX174 bacteriophage mutants altered in genes which affect the structure of the viral capsid. Progeny from cells infected simultaneously with two electrophoretic strains were found to contain DNA of either type in hybrid capsids containing wild-type and mutant proteins in varying proportions. Electrophoretically homogeneous preparations of phage with hybrid capsids were isolated. The existence of hybrid capsids was explained by the idea that the ϕX174 protein coat is assembled from preformed subunits in a pool.

7.4.5. SDS Gel Electrophoresis

Electrophoresis is extensively used to estimate molecular weights of proteins and polypeptides. The essence of the method is to treat the sample with sodium dodecyl sulfate (SDS). This detergent denatures the protein and coats the unfolded polypeptide chains more or less uniformly. Because each detergent molecule contains an ionizible sulfate group, the charge on the coated polypeptide chain is almost completely the result of the adsorbed SDS, thereby imparting nearly the same surface charge density to all polypeptide chains regardless of composition. Furthermore, the negative charges on the adsorbed detergent repel each other strongly, thereby converting the peptide into a relatively stiff rod. This material is then subjected to electrophoresis on a retarding gel, such as polyacrylamide, which then separates polypeptides on the basis of rod length or molecular weight. The low-molecular-weight peptides are less retarded than those with high molecular weight. The mobility is approximately a linear function of the logarithm of the molecular weight, as illustrated in Fig. 19. This is in no sense an absolute method

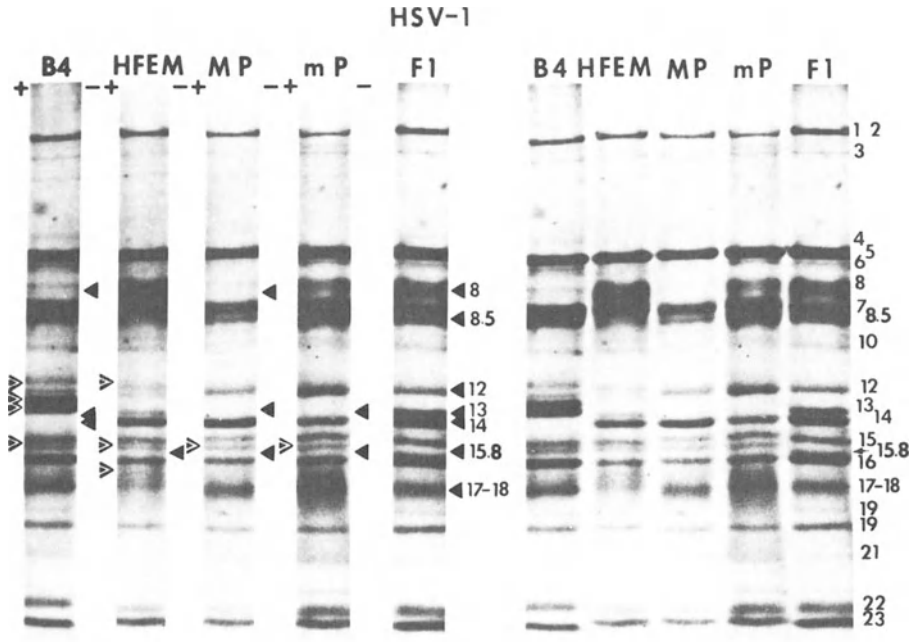


Fig. 18. Autoradiograms of a polyacrylamide gel slab containing electrophoretically separated polypeptides of HSV-1 (F1), HSV-1 (mP), HSV-1 (MP), HSV-1 (HFEM), and HSV-1 (13VB4) virions. The entire autoradiogram is shown on the right, together with the current designations of polypeptides of HSV-1 (F1). To facilitate comparisons, the autoradiograms of each gel are also shown on the left. The arrows on the (−) side of each gel pinpoint the position of HSV-1 (F1) polypeptides absent from the various HSV-1 strains tested. The arrows on the (+) side indicate the polypeptides for which there is no electrophoretic analogue in the HSV-1 (F1) virions. The arrows against the gel containing the electrophoretically separated HSV-1 (F1) polypeptides pinpoint all the polypeptides whose electrophoretic analogue is absent in one or more of the HSV-1 strains tested. From Heine *et al.* (1974). Published with the permission of the copyright owner, American Society for Microbiology.

of determining molecular weights. The mobility of any particular peptide in SDS gel electrophoresis depends on the retarding effect of the particular gel used, as well as on the molecular weight and composition of the peptide in study. Thus glycoproteins show abnormal mobility. In practice, it is desirable to add at least two markers, one of low molecular weight and one of high molecular weight known from independent experiments such as sedimentation equilibrium or amino acid sequencing, to the sample under investigation, as illustrated in Fig. 19. Then the molecular weight of the unknown or unknowns can be

interpolated using the relationship just mentioned. These methods are even more useful and more reliable for the separation of oligo- and polynucleotides which, being polyanions, need no SDS.

Valuable information beyond molecular weight determination can be obtained by this method. As an example, it has been used recently to classify orthopox viruses, an important matter in the campaign to eradicate smallpox (Harper *et al.*, 1979). HeLa cells were labeled with [^{35}S]methionine 16 hr after infection, and the late intracellular polypeptides were then analyzed by SDS polyacrylamide gel electrophoresis. Components were detected by autoradiography. From the patterns obtained, 24 viruses could be arranged in four main groups.

Gel electrophoresis is used extensively to estimate the molecular weight of DNA fragments. Because of the phosphates in the backbone, the DNA threads do not require treatment with SDS. However, caution must be exercised. Zeiger *et al.* (1972) demonstrates that base composition as well as nucleotide length affects the mobility on polyacrylamide gels. Nucleic acid fragments with low guanine-cytosine content migrated more slowly on such gels than fragments of comparable molecular weight with a higher content of those two bases. Thomas and Davis (1975) used electrophoresis in agarose gels in an investigation involving the mapping of the *Eco*RI restriction sites on the DNA of the bacteriophage λ. They found that the relative electrophoretic mobilities

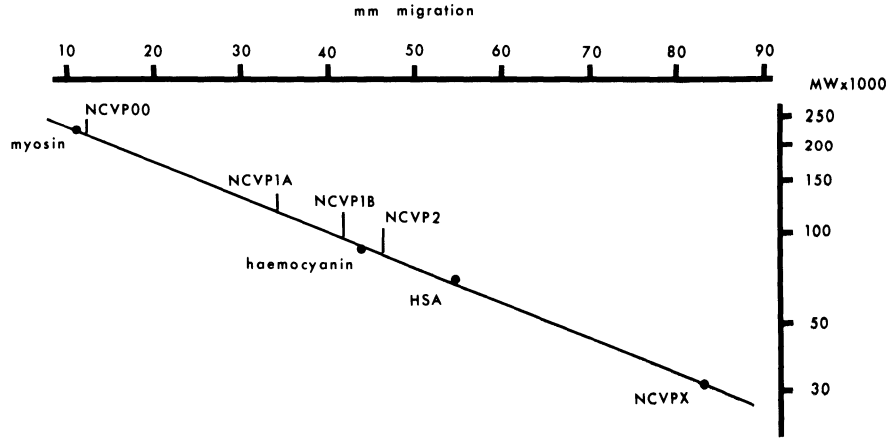


Fig. 19. Relation between mobility and molecular weight obtained on 6% acrylamide gels containing 5 M urea of three standards, myosin, hemocyanin, and human serum albumin, in comparison with poliovirus-infected cytoplasms. From Abraham and Cooper (1975). Published with the permission of the copyright owner, Cambridge University Press.

in agarose gels agree with the lengths of the DNA fragments as determined by electron microscopy. They reported further that, in agreement with the finding of Zeiger *et al.*, this is not true for electrophoresis on polyacrylamide gels.

7.5. Electrofocusing

Electrofocusing is a useful variant of electrophoresis. When an ampholyte such as a protein is dissolved in a solution in which there is a pH gradient, it will always migrate toward the position in the gradient where the pH is that of the isoelectric point of the protein. This method has been used since 1955 to separate ampholytes with different isoelectric points. Two problems are associated with it. One is to stabilize the system against convection. This can be done with a sucrose gradient or a gel. A more serious problem is the establishment and maintenance of a stable pH gradient. A clever method for accomplishing this is now available. The principle of this method can be explained by considering the properties of a dicarboxylic and of a dibasic amino acid.

If a dicarboxylic amino acid such as aspartic acid is dialyzed exhaustively against pure water, it will eventually come to an equilibrium state in which undissociated water, hydrogen ion, hydroxyl ion, and zwitterion alone are present. The pH at this equilibrium must be low enough so that each of the two carboxyl groups is approximately 50% ionized. This will occur near the isoelectric point, pH 2.77, but not precisely at that point. The reason is that at low pH the hydrogen ion concentration greatly exceeds the hydroxyl ion concentration, but electrical neutrality must be preserved in the isoionic state. This can happen only when the amino acid has a slight average net negative charge to balance the excess of the hydrogen ion concentration over the hydroxyl ion concentration. This average net negative charge can occur only at a pH value somewhat above the isoelectric point. Similarly, when a dibasic amino acid such as lysine is exhaustively dialyzed against water, it will eventually come to the isoionic state at a pH value somewhat lower than its isoelectric point, pH 9.44. If an equimolar mixture of aspartic acid and lysine is exhaustively dialyzed against water, it will come to equilibrium at some pH value intermediate between the isoionic points of the separate amino acids, somewhere near neutrality. In this solution, the aspartic acid will have a net charge of almost -1 and the lysine a charge of almost $+1$. If an electric current is now passed through this solution, the aspartic acid

in the mixture will move toward the positive electrode and the lysine will move toward the negative electrode. As soon as each gets into a region free of the other, it will come to equilibrium with water and establish the isoionic pH, where, because it is near the isoelectric point, the amino acid will then migrate only very slowly. The ultimate result will be the accumulation of aspartic acid at a low pH in the positive side of the cell and of lysine at a high pH in the negative side, with a relatively sharp pH gradient between them.

Randomly polymerized basic and acidic amino acids produce polypeptides with an almost continuous distribution of ionizable side chains and, therefore, of isoionic points, ranging from about pH 3 to about pH 10. When such a distribution of polypeptides with a wide range of isoionic points is subjected to an electric current, each polypeptide species migrates to and concentrates in a region where the pH is in the isoionic–isoelectric range for that species. When a large number of species with different isoionic points is available, the result is a stable pH gradient. When an exhaustively dialyzed solution containing several proteins in addition to these random polypeptides is subjected to electrophoresis, not only is the pH gradient established by the random polypeptides, but each protein accumulates in a band centered about the pH value corresponding to its isoelectric point. By diffusion, the band tends to broaden, but the electric field constantly tends to bring the diffused molecules back toward the center of the band. The result is sharp, well-separated bands affording excellent resolution. A limitation of this method is that not all of the resolved bands necessarily represent distinct components. The resolution is very sensitive to isoelectric points. If, for example, there is slight variation in the number of carboxyl groups in the amide form, resolvable bands could appear by the electrofocusing technique, but such minor differences might not seriously affect the structure or the function of the protein.

An interesting modification of this technique, described by O'Farrell (1975), has been applied in the study of viruses. The modified method involves electrofocusing in a gel, usually in the presence of a denaturing agent such as urea followed by SDS gel electrophoresis at right angles. This method separates proteins on the basis of both charge (isoelectric point) and molecular weight. The sensitivity is such that about 1100 different components were found in extracts of *E. coli*. O'Farrell (1975) also used the method to detect a missense mutation in gene 32 of T4 bacteriophage. The same method was used later (O'Farrell and Goodman, 1976) to study the capsid proteins of simian virus 40. One major protein with an isoelectric point at pH 6.8 and a molecular weight of about 47,000 constituted 90% of the material. In

addition, five minor components were identified, differing slightly from the major component in isoelectric point and molecular weight. Two of them were shown to be phosphorylated major component. A temperature-sensitive mutant was found to have both major and minor components shifted in isoelectric point from the proteins of the type strain.

7.6. Continuous-Flow Electrophoresis

Finally, it is necessary to discuss continuous-flow electrophoresis on paper. An appropriate experimental arrangement is to suspend a sheet of paper saturated with buffer in a vertical position. Contact with electrode vessels is made on the two vertical edges of the paper. If a gentle stream of a protein solution is applied to the paper at the top, it will tend to flow straight down under gravity. However, when an electric current is passed through the paper, negatively charged macromolecules will take a diagonal path in the direction of the positive electrode and positively charged macromolecules will take a diagonal path in the direction of the negative electrodes. In this manner, substances of different mobilities can be collected at different positions along the bottom of the paper. An illustration of the paths taken by various components is shown in Fig. 20. Since this is a continuous process, appreciable amounts of material can be separated.

7.7. Immunoelectrophoresis

Immunoelectrophoresis is a combination of electrophoretic migration in a gel and the subsequent location of antigens by precipitation

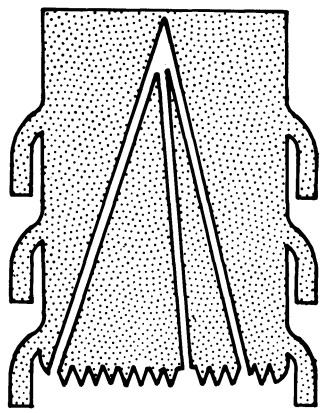


Fig. 20. Diagramatic representation of the path taken by three solutes in continuous-flow electrophoresis. A gentle stream of solutes is applied at the top. The vertical edges of the paper moistened with electrolyte solution are attached to electrodes. The solutes follow diagonal paths depending on gravity flow and the rate and direction of migration in the field. The various components can be collected at the bottom.

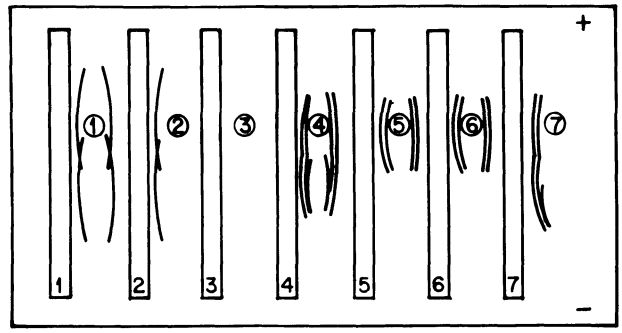


Fig. 21. Immunoelectrophoresis pattern of BMV and related antigens. The antigen wells, numbering from the left, contained the following: 1, crude juice from BMV-infected Moore barley; 2 and 3, crude juice from healthy Moore barley; 4, BMV zone; 5, antigen 2; 6, antigen 1; 7, crude juice from BMV-infected Moore barley. Troughs 1 and 2 contained undiluted healthy barley antiserum; the remaining troughs were filled with undiluted BMV antiserum. Buffer consisted of 0.016 M veronal and 0.014 M sodium chloride at pH 8.0. Voltage 13.7 V/cm; current 7 ma; time of electrophoresis 20 hr. Adapted from Hamilton (1961).

with antiserum. The technique is described by Grabar (1958) and by Williams and Grabar (1955). As illustrated in Fig. 21, Hamilton (1961) applied immunoelectrophoresis to the investigation of the soluble antigens associated with brome mosaic virus. Wells cut into thin agaragar gels on a glass plate were filled with antigens, and electrophoresis was carried out. After electrophoresis, troughs were cut in the gel parallel to the direction of flow of the current, and antisera were placed in them. From this point on, the technique involved immunodiffusion and the formation of zones of precipitate where antigen and antibody came together in the range of equivalent concentrations.

8. OSMOTIC PRESSURE

One of the oldest physical methods for determining molecular weight is osmotic pressure. It is still used on occasion in studies on proteins, including viral coat proteins. An attractive feature of this method is that it is thermodynamic and therefore free of the ambiguities associated with the arbitrary choice of models. When a solution is separated from a pure solvent by a semipermeable membrane, that is, a membrane permeable to solvent molecules but not to solute molecules, solvent molecules will diffuse through the membrane into the solution. In the absence of restraints, this process will continue until all of the pure solvent has diffused into the solution. However, the transfer of

solvent across the membrane can be brought to a halt by applying pressure on the solution. The pressure just sufficient to prevent any net flow of solvent across the membrane is defined as the osmotic pressure, π_0.

The transfer of solvent to a solution at a higher pressure can be accomplished, conceptually, in two steps. First, solvent can be added to solution at constant temperature at the constant pressure of the solvent. This represents a change in the activity, a_1, of the solvent. When the change is infinitesimal, the change in chemical potential is $RT\,d\ln a_1$. In the second step, the pressure on the newly diluted solution can be raised infinitesimally. The change in chemical potential for this step is $\overline{V}_1 dP$, where $\overline{V}_1$ is the partial molal volume of solvent and P is the pressure. The total change in chemical potential is the sum of these. If the process is carried out reversibly, that sum must equal zero. Thus equation (61) can be written.

$$\overline{V}_1 dP + RT\,d\ln a_1 = 0 \tag{61}$$

When equation (61) is solved for dP, equation (62) results.

$$dP = -\frac{RT}{\overline{V}_1}\,d\ln a_1 \tag{62}$$

At constant pressure and constant temperature, the activities of solvent and solute in a binary solution are related by equation (63), an aspect of the Gibbs-Duhem relationship of thermodynamics.

$$d\ln a_1 = -(n_2/n_1)\,d\ln a_2 \tag{63}$$

In this equation, n_1 and n_2, respectively, represent the number of moles of solvent and solute. When equation (63) is substituted into equation (62) and when $n_1\overline{V}_1$ is set equal to v_1, the total volume of solvent, equation (64) results.

$$dP = \frac{RT}{v_1} n_2\,d\ln a_2 \tag{64}$$

$$d\ln a_2 = da_2/a_2 = dn_2/n_2 \tag{65}$$

The right member of equation (65) is an accurate substitute for the central member for very dilute ideal solutions. Substitution into equa-

tion (64) yields

$$dP = \frac{RT}{v_1} dn_2 \tag{66}$$

When this is integrated across a membrane separating a solvent at atmospheric pressure, where the number of moles of solute is zero, from a solution at osmotic equilibrium, where the osmotic pressure is π_0 higher than atmospheric and where there are n_2 moles of solute in a volume v_1 of solute, equation (67) is obtained.

$$\pi_0 = (n_2/v_1)RT \tag{67}$$

When M_2 is the molecular weight of solute and ρ_1 is the density of solvent the concentration of solute, c, expressed as grams per gram of solvent, is $n_2M_2/(\rho_1/v_1)$. From this it follows that

$$n_2/v_1 = \rho_1 c/M_2 \tag{68}$$

Equation (69) is obtained by substituting this value of n_2/v_1 into equation (67).

$$\pi_0 = \frac{\rho_1 cRT}{M_2} \tag{69}$$

Equation (69) shows that for an ideal dilute solution the osmotic pressure, π_0, divided by the concentration expressed in terms of grams per gram of solvent is inversely proportional to the molecular weight of the solute. When Stanley (1935) first crystallized tobacco mosaic virus, he attempted to measure the osmotic pressure of a solution of the virus and found a value within experimental error of zero. Using the ideas inherent in equation (69), he inferred that the mass of the virus was at least several million daltons.

Because of their high molecular weights, solutions of proteins are usually dilute in terms of moles per unit volume. However, they are rarely ideal. For real solutions, additional terms must be added in higher powers of concentration. For most protein solutions, it is sufficiently accurate to add a single term involving c^2, as illustrated in

$$\pi = \rho_1 RT\,(c/M_2 + A_2'c^2) \tag{70}$$

For highly accurate data, it is sometimes appropriate to add a third term in c^3. In equation (70), the constant A_2' is called the second virial coefficient. It can be evaluated experimentally by plotting π_0/c vs. c. The equation predicts that a straight line should be obtained with intercept equal to $\rho_1 RT/M_2$ and with slope $\rho_1 RTA_2'$. The slopes of such graphs usually turn out to be positive for osmotic pressure data obtained with proteins. This is illustrated by data obtained with succinylated cucumber virus 4 protein, as shown in Fig. 22 (Frist *et al.*, 1965). This is a virus of the TMV (tobamo) group. The most important aspect of such a graph is that the intercept is inversely proportional to the molecular weight; the value for M_2 is 21,500 in the experiment shown in Fig. 22.

This estimate of the molecular weight is somewhat higher than that obtained from sedimentation and diffusion measurements, 19,000, a value closer to that usually found for the protein subunits of tobacco mosaic virus and closely related viruses. While experimental error might account for some of the difference, it is more probable that the protein preparation contained, in addition to succinylated monomeric units, a small amount of aggregated material. Equation (67) shows that osmotic pressure is directly proportional to the number of moles of solute per unit volume. Equation (68) shows that the calculated molecular weight is proportional to the concentration expressed in grams per unit volumes divided by moles per unit volume. When a solution contains several solutes with different molecular weights, the molecular weight calculated from the osmotic pressure is a number

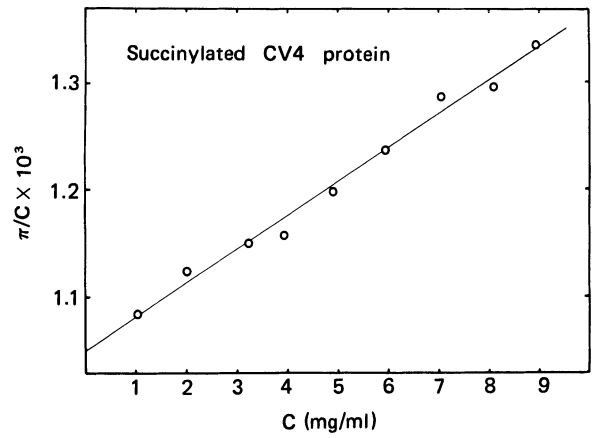


Fig. 22. Osmotic pressure data for succinylated CV4 protein expressed in atmospheres. From Frist *et al.* (1965). Reproduced with the permission of the copyright owner, Acamdemic Press, Inc.

average molecular weight, an average obtained by dividing the total weight of solute by the total number of moles of solute. Osmotic pressure measurements are most sensitive to solutes of low molecular weight. Therefore, the osmotic pressure method is best adapted to study of low-molecular-weight proteins. The method becomes insensitive when the molecular weight exceeds a few hundred thousand.

A great deal of theoretical thinking has gone into the interpretation of the second virial coefficient (Scatchard, 1946; Lauffer, 1966). The main contributions are interaction of solute molecules with each other, an excluded-volume effect, interaction of solute molecules with other components, for example, hydration, and an effect of electric charge, usually described as the Donnan effect. For most proteins at pH values significantly different from the isoelectric point, the Donnan effect is overwhelmingly the largest contributor to the second virial coefficient. This contribution is always positive, that is, it leads to a higher value of π_0/c at finite protein concentrations than the value at infinite dilution.

There are times, however, when other contributions to the second virial coefficient overwhelm the positive Donnan contribution. This is illustrated in the data in Fig. 23, showing osmotic pressure data for tobacco mosaic virus protein (Banerjee and Lauffer, 1966). In this case, the intercept is consistent with a molecular weight of 53,000. Since the molecular weight of the protein subunit is known from amino acid analyses to be close to 17,500, this result is one of the reasons for believing that TMV protein can exist in the form of stable trimers. In this case, however, the second virial coefficient is negative, and it is more negative at 15.8° than at lower temperatures. This has been interpreted to mean that the interaction between protein molecules is much greater than the Donnan term and increases in negative magnitude as the temperature is increased. An extreme aspect of protein–protein interaction is actual combination or polymerization. Thus the data displayed in this figure show that the polymerization of TMV protein increases with increasing temperature; it is endothermic and therefore entropy driven.

9. LIGHT SCATTER

9.1. Theory

A closely related method, related for reasons which will be apparent presently, is light scatter. If I_0 is the intensity of an incident

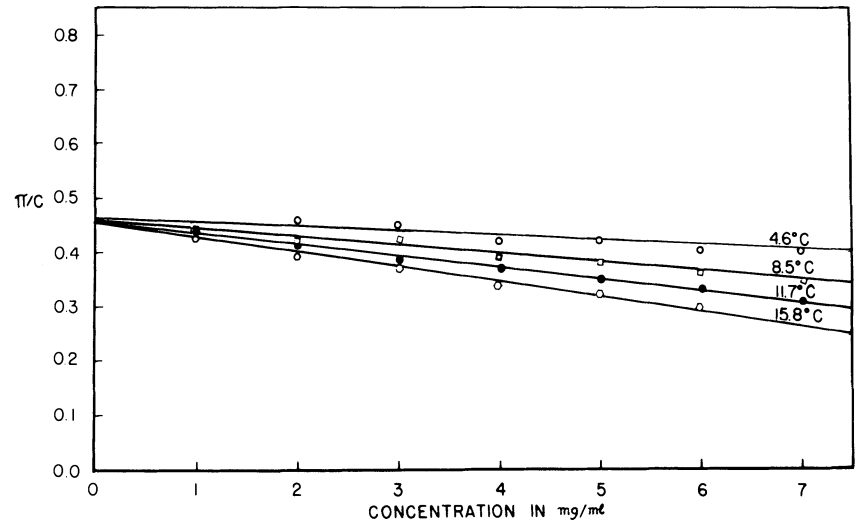


Fig. 23. Effect of temperature on osmotic pressure of TMV protein in phosphate buffer, pH 7.5, ionic strength 0.067. π is the osmotic pressure in centimeters of water, and c is the protein concentration in milligrams per milliliter. From Banerjee and Lauffer (1966). Reproduced with the permission of the copyright owner, the American Chemical Society.

beam of light impinging on a solution of volume, V, the intensity, I', scattered in a direction θ from the incident beam is given by equation (71), where K is defined by equation (72).

$$I' = 2\pi^2(1 + \cos^2\theta)I_0VK \tag{71}$$

$$K = n^2(dn/dg)^2kTg/\lambda^4r^2(d\pi_0/dg) \tag{72}$$

In equation (72), n is the refractive index of the solution, g is the concentration expressed in terms of weight per unit volume of solution, k is the Boltzmann constant, λ is the wavelength of light, and r is the distance from the center of scattering to where the intensity is measured. Equations (71) and (72) are derived from electromagnetic theory and from the Einstein–Debye theory for the fluctuation of the polarizability of liquids. They are valid for the simplest case in which solute particles have no dimension greater than approximately $\lambda/20$. A convenient source for this derivation is Tanford (1961).

It is a matter of common experience that light is not deflected unless there is a difference between the refractive index of the object and the surrounding fluid. This translates into a difference in polarizability. Solutions can be considered to be made up of tiny elements of

volume in which the refractive index fluctuates about the mean value. This results from fluctuations in density in the case of pure liquids, and from this and fluctuations in concentration in the case of solutions. The Einstein–Debye treatment relates the fluctuation in concentration to $(d\pi_0/dg)$. This effect is noramlly much greater than the fluctuation in density of the solvent. It is through the $(d\pi_0/dg)$ term that light scatter theory is closely related to osmotic pressure theory; indeed, this is the basis for considering light scatter a thermodynamic method.

9.2. Turbidity

A convenient way to study light scatter, one used extensively in research on the self-assembly of viral proteins, is to transform equations (71) and (72) into one involving the turbidity, τ, of the solution. Turbidity is defined by equation (73).

$$-dI/dx \equiv \tau I \tag{73}$$

When a beam of light of cross-sectional area A is scattered by a volume of solution, V,

$$\int \tau\, dx = \tau V/A = -\int dI/I = \int_0^{\pi} I' 2\pi r \sin\theta r d\theta / I_0 A \tag{74}$$

The extreme right-hand member of equation (74) relates the fractional loss of intensity to the intensity of light scattered, integrated over all values of θ at the surface of a sphere r centimeters away from the scattering center, divided by the product of the incident intensity, I_0, and the area, A, through which the incident beam enters the solution. When the values of I' and of K given by equations (71) and (72) are introduced and the integration is performed, equation (75) results.

$$\tau = (32/3)\pi^3 n^2 (dn/dg)^2 kTg/\lambda^4 (d\pi_0/dg) \equiv HRTg/(d\pi_0/dg) \tag{75}$$

The g in equation (75) is approximately equal to $\rho_1 c$ found in equation (70). This approximation is good for dilute solutions. When this substitution is made in equation (70) and when A_2 is defined as A_2'/ρ_1, equation (76) results.

$$\pi_0 = RTg/M_2 + \mathrm{RTA}_2 g^2 \tag{76}$$

$$d\pi_0/dg = RT/M_2 + 2RTA_2 g \tag{77}$$

When this is substituted into equation (75) and rearranged,

$$Hg/\tau = 1/M_2 + 2A_2g \tag{78}$$

At low values of the concentration, g, the term on the extreme right of equation (78) can be dropped. Turbidity then becomes directly proportional to the product of the concentration of solute and the molecular weight, with H as the proportionality constant. When more than one solute is present, the total turbidity is the sum of the contributions from each of the solutes. It is proportional to the sum of the products of concentration times molecular weight of each solute. The average molecular weight for such a mixture is proportional to this sum divided by the total concentration. This gives a kind of average called a weight average, which is quite different from the number average obtained from osmotic pressures studies. This type of average is sensitive to high-molecular-weight components. Indeed, turbidity itself is more sensitive to high-molecular-weight than to low-molecular-weight constituents. This, therefore, makes light scatter methods most useful for investigating proteins with molecular weights higher than those amenable to study by osmotic pressure. However, as will be seen later, a complication is encountered when the solute particles become much larger than $\lambda/20$. For such particles, equation (78) is no longer valid.

Light scatter has been used extensively to study the polymerization of viral coat proteins, especially tobacco mosaic virus protein. When turbidity is measured, it is possible to use a spectrophotometer, provided only that a wavelength of light is chosen at which the protein does not absorb. Such instruments are normally calibrated in terms of optical density, OD. Optical density readings are readily converted to turbidity; $\tau = 2.303$ OD. Figure 24 shows data obtained in this manner for the polymerization of tobacco mosaic virus coat protein at various values of pH (Lauffer and Shalaby, 1980). The wavelength of the light used in these studies was 320 nm. The principal product formed in this polymerization, especially at the lower pH values, is a rod with a diameter of about 18 nm and with variable lengths. The diameter of the rod is slightly more than $\lambda/20$ but not sufficient to introduce a large departure from equation (78). A rod with a diameter of 18 nm and a length of 18 nm would have a mass slightly in excess of 2 million daltons. This is about the limit for the application of equation (78). Such a mass corresponds to an OD of approximately 0.04. Thus interpretation of the data of Fig. 24 in terms of equation (78) must be confined to the lower range of values of OD. To interpret these data in

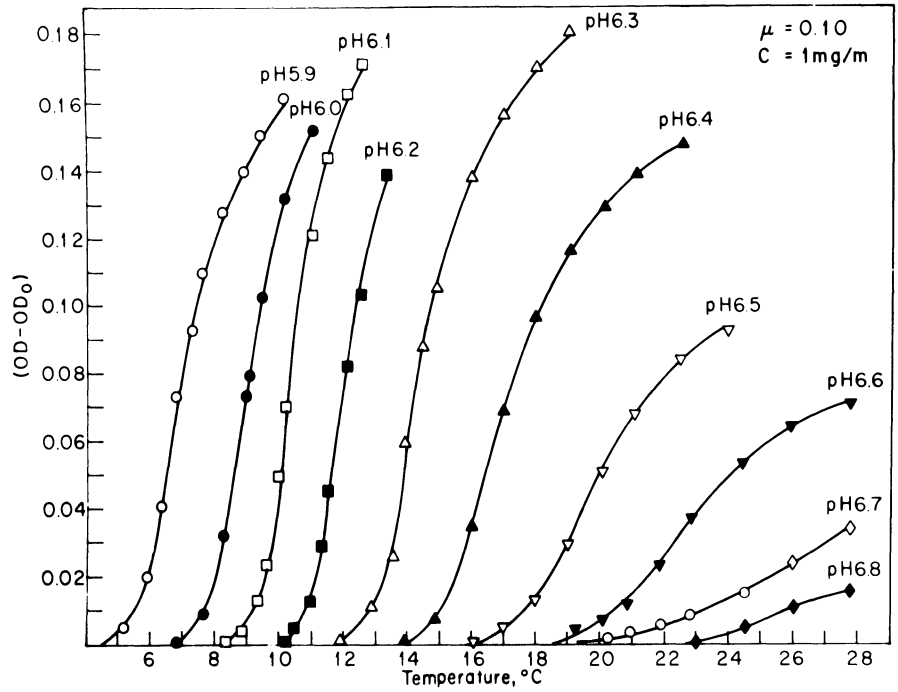


Fig. 24. Optical density (OD – OD_0) of TMV protein solutions as a function of temperature at various pH values. From Lauffer (1978). Reprinted with the permission of the copyright owner, Elsevier North-Holland.

terms of average particle weight, it is necessary to know the value of the proportionality contant, H. The factors lumped together in this constant are shown in equation (75), the most important of which is the specific refractive increment, dn/dg, of the protein at the wavelength chosen. How this was obtained is described by Smith and Lauffer (1967); the value of H was calculated to be 4.23×10^{-5} (cm/g)^2.

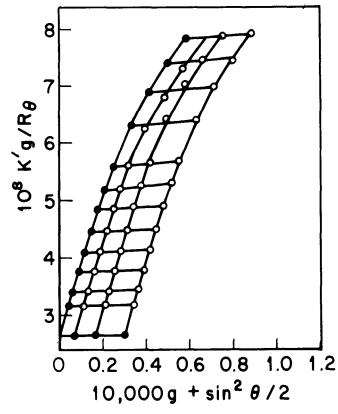


Fig. 25. Light scattering of TMV solutions: 0.01 M phosphate buffer, pH 7.1; M = 38,200,000 L = 3200 Å. O, Experimental data, ●, extrapolated points. Redrawn from data of Boedtker and Simmons (1958).

This method has also been used to follow the self-assembly of several other viral proteins, recently by Erickson and Bancroft (1978) in studies on papaya mosaic virus protein. It polymerizes at pH 8.0 from 14 S at 5°C to 25 S at 25°C and dissociates again on cooling. Like tobacco mosaic virus protein polymerization, this reaction is endothermic and therefore entropy driven. The effects of pH and of salt concentration are opposite to those found with tobacco mosaic virus protein. Turbidity measurements were also used to follow the course of reconstitution of tobacco mosaic virus from its protein and RNA (Butler and Klug, 1971; Richards and Williams, 1972).

9.3. Angular Dependence

When equation (72) is substituted into equation (71), the full expression for the intensity of the scattered beam is obtained. It is evident that this intensity is dependent on two properties of the apparatus used to measure the scattered intensity, the angle, θ, between the incident beam and the scattered beam, and the distance, r, between the detecting instrument and the scattering centers. Various kinds of apparatus are available for measuring intensity of scattered light as a function of the angle, θ. It is convenient to group the terms in combined equations (71) and (72) in a somewhat different manner, as shown in equations (79) and (80), where Av is Avogadro's number.

$$R_\theta \equiv I'r^2/I_0(1 + \cos^2\theta) \tag{79}$$

$$K' \equiv 2\pi^2 n^2 (dn/dg)^2/Av\lambda^4 \tag{80}$$

R_θ is an experimental variable in that all of the elements contained in its definition can be determined experimentally. As long as the simple theory discussed thus far is valid, R_θ should be independent, within experimental error, of the angle θ and the distance r. K' of equation (80) is merely a slightly different selection of constants from those involved in K of equation (72). When these new difinitions are substituted into combined equations (71) and (72) and when equation (77) is substituted for $d\pi_0/dg$, equation (81) results.

$$R_\theta = K'g/(1/M_2 + 2A_2g) \tag{81}$$

In the limit as g approaches zero, R_θ becomes equal to $K'gM_2$, a result very similar to that obtained for turbidity at infinite dilution. One of

the tests of the validity of the simple theory for the material under investigation is whether or not R_θ really is independent of θ within experimental error.

9.4. Scattering from Large Particles

When the solute particles have at least one dimension significantly larger than $\lambda/20$, destructive interference in the scattered light is encountered. Depending on the angle θ, the intensity of the scattered light is less than it would be without interference. The usefulness of this fact was first recognized by Guinier (1939). If I'' is defined as the intensity of light scattered with interference, in contrast with the I' of equation (71), which represents the intensity of the scattered light without interference, then, in the limit as concentration approaches zero, I'/I'' is given by

$$I'/I'' = 1 + (16/3\lambda^2)\pi^2 R_G^2 \sin^2(\theta/2) \tag{82}$$

A derivation of this equation is given in Tanford (1961). R_G in equation (82) is called the radius of gyration. For solid spherical particles, the square of the radius of gyration is equal to 3/5 the square of the radius, and for long straight rods of length L the square of the radius of gyration is equal to the square of the length divided by 12. When an extrapolation of R_θ *to* $\theta = 0$ is carried out, it is possible to evaluate both M_2 and A_2 according to equation (81). When R_θ is extrapolated to zero concentration, it is possible to evaluate R_G according to equation (82). Zimm (1948) has developed a method for making both kinds of extrapolation, illustrated by data obtained with tobacco mosaic virus as displayed in Fig. 25, the results of Boedtker and Simmons (1958). In this manner, they obtained values of 38.2 and 40.8 $\times$ 10^6 daltons for the mass and of 320 nm for the length with two different preparations of the virus.

9.5. Small-Angle X-Ray Scattering

Conceptually related to the scattering of light by particles larger than $\lambda/20$ is the scattering of X-rays by proteins in solution. When a solution of a protein is irradiated with X-rays with wavelengths near 1 Å (copper Kα radiation with $\lambda = 1.54$ Å is frequently used), the radiation will be scattered through very small angles, 2θ. The theory of

small-angle X-ray scattering is beyond the scope of the present treatment. However, it turns out that the intensity of the scattered radiation is a function of both the angle, θ, and the radius of gyration, R_G, of the protein. The final equation in the theory, an approximate equation strictly valid only as θ approaches zero, is

$$\ln I = \ln I_0 - h^2 R_G^2/3 \tag{83}$$

In this equation, I is the intensity of the light scattered at some angle θ, I_0 is the value when θ is zero, and h is $(4\pi/\lambda)\sin\theta$. When $\ln I$ is plotted against h^2, the limiting slope is $-R_G^2/3$. Values for R_G have been reported for several virions, for example, southern bean mosaic virus (Leonard *et al.*, 1951), tomato bushy stunt virus (Leonard *et al.*, 1953), tobacco necrosis virus (Leonard *et al.*, 1951, 1953), turnip yellow mosaic virus (Schmidt *et al.*, 1954), and wild cucumber mosaic virus (Anderegg *et al.*, 1961).

9.6. Intensity Fluctuation of Scattered Light

Another tool related to light scatter has been applied to the study of virions. It depends on measuring the fluctuation of the intensity of the light scattered in a particular direction by a solution of macromolecules. The term $d\pi_0/dg$ of equation (72) is related in simple light scatter theory to the mean squared fluctuation in polarizability of tiny elements of volume within the solution. Fluctuations in polarizability are related to fluctuations in refractive index which in turn are related to fluctuations in concentration. It is possible, with the use of a laser beam as a light source and appropriate detecting equipment, to measure the actual fluctuations in intensity of the scattered light. These fluctuations are related to the rate at which solute diffuses. If Γ is the rate constant for exponential decay of the amplitude of the fluctuating electric field of the scattered light, theory relates Γ to the translational diffusion coefficient of the macromolecules by

$$\Gamma = D(4\pi/\lambda)^2 n^2 \sin^2(\theta/2) \tag{84}$$

In this equation, D is the coefficient of diffusion, n is the solution refractive index, λ is the wavelength of the incident light, and θ is the scattering angle. It is also possible to determine the rotational diffusion coefficient of nonspherical particles and the flexing of nonrigid particles

by this technique. A qualitative description of the theory is given by Pusey *et al.* (1974). The method has been used to study several spherical viruses, including tomato bushy stunt virus, T7 bacteriophage (Camerini-Otero *et al.*, 1974), and tobacco mosaic virus (Cummins *et al.*, 1969, Fujime, 1970; Wada *et al.*, 1971). D_w^{20} was found to be 3.26×10^{-8} cm^2/sec (Wada *et al.*, 1971), a value 20% lower than that found by Schramm and Bergold (1947) by the usual method.

10. THEORY OF ELECTRON MICROSCOPY

10.1. Historical Review

One of the most widely used of all physical techniques in the study of viruses is electron microscopy. Actual results have been presented by Williams and Fisher (1974). The present treatment is confined to an elementary discussion of the properties of light which make it impossible to resolve most viruses with even the best light microscopes, the properties of electrons which make electron microscopy feasible, and some of the limitations inherent in electron optics. A detailed discussion of the applications of electron microscopy to virology is the subject matter of the next chapter.

All except the largest viruses have at least one dimension smaller than 200 nm or 2000 Å. While it would be easy to enlarge an optical image sufficiently, it is still not possible to see most viruses. The reason is not lack of magnification but lack of resolution. One of the fundamental properties of light is that it is diffracted by gratings and also by lenses. This fundamental property can be explained only in terms of the wave theory and is the basic reason why, during the nineteenth century, that theory supplanted the corpuscular theory previously proposed by Newton. A consequence of diffraction is that an image of a point source of light does not appear as a point but as a disk surrounded by faint concentric rings. The disk in the image plane is called a circle of confusion. Whenever two point sources of light are so close together that their circles of confusion overlap by more than approximately half, it is practically impossible to determine by inspecting the image whether the light came from one or from two point sources. In other words, the two points can not be resolved. The minimum resolvable distance, d, depends on the wavelength of light and the numerical aperture, N.A., of the lens. The N.A. of a microscope objective is approximately the ratio of the radius of the lens to the distance of the object from the edge of the lens, multiplied by the refractive index of

the material between the lens and the object. The formula relating these variables is given by

$$d = \frac{5/4\ \lambda \sin \alpha_1}{2 \tan \alpha_1 \text{ N.A.}} \simeq \tfrac{1}{2}\ \lambda \tag{85}$$

In this equation, λ is the wavelength of the light, and α_1 is the angle between the center and the edge of the objective at the image formed by the objective lens in the barrel of the microscope. Since α_1 is a very small angle, sin α_1 is almost equal to tan α_1. Furthermore, even for very good objectives, N.A. is 1.5 or less. Thus the approximation on the extreme right is obtained. For visible light of wavelength 500 nm, the minimum resolvable distance, d, is accordingly about 250 nm.

Not all of the properties of light can be explained in terms of the wave theory. The photoelectric effect requires that light be corpuscular. Thus light must have a dual nature, both wave like and corpuscular. It is propagated as corpuscles, called photons, associated with electromagnetic waves. Einstein's theory of relativity gives the energy of the photon as

$$E = mc^2 \tag{86}$$

where E is the energy, m is the relativistic mass, and c is the speed of light. Quantum theory, on the other hand, relates the energy of the photon to its frequency, ν, multiplied by Planck's constant, h.

$$E = h\nu \tag{87}$$

When these two expressions for E are equated, equation (88) results.

$$mc^2 = h\nu = hc/\lambda \tag{88}$$

When this is solved for λ, equation (89) is obtained, where P is the momentum of the photon.

$$\lambda = h/mc = h/P \tag{89}$$

deBroglie reasoned that, if photons have momentum related to the wavelength by equation (89), particles of matter, such as electrons, protons, etc., moving at high velocities, should be expected to be associated with matter waves with wavelengths also given by equation

(89). If high-velocity particles, like electrons, have matter waves, it should be possible to diffract them, just as it is possible to diffract light. Davisson and Germer (1927) proved experimentally that this is true and verified the appropriateness of equation (89).

The kinetic energy of an electron moving at velocity v is equal to $\frac{1}{2}mv^2$. The energy of an electron accelerated by a potential drop, V, is equal to Ve, where e is the electronic charge. When these are equated and solved for momentum, one obtains

$$P = mv = \sqrt{2mVe} \tag{90}$$

When this value for P is substituted into equation (89),

$$\lambda = h/\sqrt{2mVe} = \sqrt{150}/V \tag{91}$$

The expression on the right of equation (91) is obtained by substituting the appropriate numerical values for h and for the mass, m, and the charge, e, of the electron, and expressing λ in Å and V in volts. From equation (91), it can be calculated readily that if electrons are accelerated across a potential drop of 60,000 volts the associated matter waves should have a wavelength of 1/20 Å. Therefore, if electron lenses can be constructed with sufficiently high numerical apertures, and if the laws of resolution are the same as for light, expressed by equation (85), then it should be possible to produce electron microscopes with vastly greater resolving power than light microscope. Electrons can be focused, either by axially symmetrical electrostatic fields or by axially symmetrical magnetic fields such as those obtained in the center of a solenoid. All of these things taken together establish the physical basis for the electron microscope.

10.2. Problems

Many problems are encountered in electron microscopy, not the least of which is that all matter, including gas, is opague to electrons. A major consequence is that in the electron microscope the electron path must be the best vacuum available. This normally requires that the sample be completely dried. A second consequence is that the support for the object, the counterpart of the microscope slide, must be an extremely thin film to minimize its opaqueness to electrons. Even so, the contrast between supporting film and object is poor when the object

is very small. Ingenious techniques have been devised to minimize all of these difficulties. A final problem is that it is very difficult to produce electron lenses of good optical quality. Spherical abberation is encountered and numerical apertures are low. The net result is that the practical resolution of electron microscopes is much poorer than that implied by equation (91). The best resolution achieved in normal practice is a few angstroms. This, however, is good enough not only to allow for beautiful pictures of viruses, but even to provide considerable structural detail. Even unwound nucleic acid threads are micrographed in many laboratories (as evidenced by the following chapter).

11. REFERENCES

Abraham, G., and Cooper, P. D., 1975, Poliovirus polypeptides examined in more detail, *J. Gen. Virol.* **29**:199.

Allard, H. A., 1916, Some properties of the virus of the mosaic disease of tobacco, *J. Agr. Res.* **6**:649.

Anderegg, J. W., Giel, P. H., Beeman, W. W., and Kaesberg, P., 1961, An x-ray scattering investigation of wild cucumber mosaic virus and a related protein, *Biophys. J.* **1**:657.

Anderer, F. A., Wittmann-Leibold, B., and Wittmann, H. G., 1965, Weitere Untersuchungen zur Aminosäuresequenz des Proteins in Tabakmosaikvirus, *Z. Naturforsch.* **20b**:1203.

Ansevin, A. T., and Lauffer, M. A., 1959, Native tobacco mosaic virus protein of molecular weight 18,000, *Nature (London)* **183**:1601.

Aposhian, H. V., 1975, Pseudovirions in animals, plants and bacteria, in: *Compr. Virol.* **5**:155.

Ball, E. M., 1964, Serology: Techniques used in plant virus research, in: *Plant Virology* (M. K. Corbette and H. D. Sissler, eds.), pp. 235–252, University of Florida Press, Gainesville, Fla.

Banerjee, K., and Lauffer, M. A., 1966, Polymerization-depolymerization of tobacco mosaic virus protein. VI. Osmotic pressure studies of early stages of polymerization, *Biochemistry* **5**:1957.

Banerjee, K., and Lauffer, M. A., 1971, Role of cation and anion in the polymerization and depolymerization of tobacco mosaic virus protein, *Biochemistry* **10**:1100.

Beijerinck, M. W., 1898, Ueber ein Contagium vivum fluidum als Ursache der Fleckenkrankheit der Tabaksblätter, *Verh. K. Akad. Wet. Amsterdam Ald. II* **6**(5):1–21.

Bier, M., ed., 1959, *Electrophoresis*, Academic Press, New York.

Bland, J. O. W., 1928, Filter and centrifuge experiments with guinea-pig vaccinia virus, *Br. J. Exp. pathol.* **9**:283.

Boedtker, H., and Simmons, N. S., 1958, The preparation and characterization of essentially uniform tobacco mosaic virus particles, *J. Am. Chem. Soc.* **80**:2550.

Brakke, M. K., 1951, Density gradient centrifugation: A new separation technique, *J. Am. Chem. Soc.* **73**:1847.

Brakke, M. K., 1960, Density gradient centrifugation and its application to plant viruses, *Adv. Virus. Res.* **7**:193.

Bull, H. B., 1971, *An Introduction to Physical Biochemistry*, F. A. Davis, Philadelphia.

Butler, P. J. G., and Klug, A., 1971, Assembly of the particles of tobacco mosaic virus from RNA and disks of proteins, *Nature* (*London*) *New Biol.* **229**:47.

Camerini-Otero, R. D., Pusey, P. N., Koppel, D. E., Schaefer, D. W., and Franklin, R. M., 1974, Intensity fluctuation spectroscopy of laser light scattered by solutions of spherical viruses: R17, Q, BSV, PM2 and T7. II. Diffusion coefficients, molecular weights, solvation and particle dimensions, *Biochemistry* **13**:960.

Caspar, D. L. D., 1963, Assembly and stability of the tobacco mosaic virus particle, *Adv. Protein Chem.* **18**:37.

Chu, P. W. G., and Francki, R. I. B., 1979, The chemical subunit of tobacco ringspot virus coat protein, *Virology* **93**:398.

Cooper, P. D., 1977, Genetics of picornaviruses, in: *Comprehensive Virology*, Vol. 9 (H. Fraenkel-Conrat and R. Wagner, eds.), pp. 133–207, Plenum Press, New York.

Craig, L. C., 1950, Partition chromatography and counter-current distribution, *Anal. Chem.* **22**:1346.

Craigie, J., 1932, The nature of the vaccinia flocculation reaction, and observations on the elementary bodies of vaccinia, *Br. J. Exp. Pathol.* **13**:259.

Cummins, H. Z., Carlson, F. D., Herbert, T. J., and Woods, G., 1969, Translational and rotational diffusion constants of tobacco mosaic virus from Rayleigh linewidths, *Biophys. J.* **9**:518.

Davisson, C. J., and Germer, L. H., 1927, Diffraction of electrons by a crystal of nickel, *Phys. Rev.* **30**:705.

Durham, A. C. H., 1972, Structures and roles of the polymorphic forms of tobacco mosaic virus protein. I. Sedimentation studies, *J. Mol. Biol.* **67**:289.

Edsall, J. T., and Wyman, J., 1958, *Biophysical Chemistry*, Vol. 1, Academic Press, New York.

Elford, W. J., 1931, A new series of graded collodion membranes suitable for general bacteriological use, especially in filterable virus studies, *J. Pathol. Bacteriol.* **34**:505.

Erickson, J. W., and Bancroft, J. B., 1978, The self-assembly of papaya mosaic virus, *Virology* **90**:36.

Frist, R. H., Bendet, I. J., Smith, K. M., and Lauffer, M. A., 1965, The protein subunit of cucumber virus 4; degradation of viruses by succinylation, *Virology* **26**:558.

Fujime, S., 1970, Quasi-elastic light scattering from solutions of macromolecules. I. Doppler broadening of light scattered from solutions of tobacco mosaic virus particles, *J. Phys. Soc. Jpn.* **29**:416.

Gafford, L. G., Mitchell, E. B., Jr., and Randall, C. C., 1978, Sedimentation characteristics and molecular weights of three poxvirus DNAs, *Virology* **89**:229.

Gish, D. T., Ramachandran, L. K., and Stanley, W. M., 1958, Studies on the amino acid sequence of tobacco mosaic virus (TMV) protein. I. Fractionation of products of tryptic hydrolysis by counter-current distribution, *Arch. Biochem. Biophys.* **78**:433.

Grabar, P., 1958, The use of immunochemical methods in studies on proteins, *Adv. Protein Chem.* **13**:1.

Guinier, A., 1939, Diffraction of x-rays of very small angles—Application to the study of ultramicroscopic phenomena, *Ann. Phys.* [*11*] **12**:161.

Hamilton, R. I., 1961, Properties of brome mosaic virus and its related antigens, *Virology* **15**:425.

Harms, E., Rohde, W., Bosch, F., and Scholtissek, C., 1978, Biochemical studies on influenza viruses. II. Assignment of gene functions to RNA segments 5, 7 and 8 of fowl plague virus and virus N, *Virology* **86**:413.

Harper, L., Bedson, H. S., and Buchan, A. B., 1979, Identification of orthopoxviruses by polyacrylamide gel electrophoresis of intracellular polypeptides. 1. Four major groupings, *Virology* **93**:435.

Haschemeyer, R. H., and Haschemeyer, A. E. V., 1973, *Proteins: A Guide to Study by Physical and Chemical Methods*, John Wiley, New York.

Heine, G. W., Honess, R. W., Cassai, E., and Roizman, B., 1974, Proteins specified by herpes simplex virus. XII. The virion polypeptides of type 1 strains, *J. Virol.* **14**:640.

Hughes, T. P., Parker, R. F., and Rivers, T. M., 1935, Immunological and chemical investigations of vaccine virus. II. Chemical analysis of elementary bodies of vaccinia, *J. Exp. Med.* **62**:349.

Hutchison, C. A., III, Edgell, M. H., and Sinsheimer, R. L., 1967, The process of infection with bacteriophage ϕX174. XII. Phenotypic mixing between electrophoretic mutants of ϕX174, *J. Mol. Biol.* **23**:553.

Ikemura, T., Okada, K, and Ozeki, H., 1978, Clustering of transfer RNA genes in bacteriophage BF23, *Virology* **90**:142.

Iwanowski, D., 1892, Über die Mosaikkrankheit der Tabakspflanze, *Bull. Acad. Imp. Sci. St. Petersbourg* **3**:67.

Kramer, E., and Wittmann, H. G., 1958, Elektrophoretische Untersuchungen der A-Proteine dreier Tabakmosaikvirus-Stämme, *Z. Naturforsch.* **13b**:30.

Laemmli, U. K., 1970, Change of structural proteins during the assembly of the head of bacteriophage T4, *Nature (London)* **227**:680.

Lauffer, M. A., 1938, The molecular weight and shape of tobacco mosaic virus protein, *Science* **87**:469.

Lauffer, M. A., 1944*a*, The size and shape of tobacco mosaic virus particles, *J. Am. Chem. Soc.* **66**:1188.

Lauffer, M. A., 1944*b*, The influence of concentration upon the sedimentation rate of tobacco mosaic virus, *J. Am. Chem. Soc.* **66**:1195.

Lauffer, M. A., 1946, Viscometry in biochemical investigation, in: *Currents in Biochemical Research* (D. E. Green, ed.), pp 241–260, Interscience, New York.

Lauffer, M. A., 1961, Theory of diffusion in gels, *Biophys. J.* **1**:205.

Lauffer, M. A., 1966, Polymerization–depolymerization of tobacco mosaic virus protein. V. Osmotic pressure theory for hydrated protein, *Biochemistry* **5**:1952.

Lauffer, M. A., 1975, *Entropy-Driven Processes in Biology: Polymerization of Tobacco Mosaic Virus Protein and Similar Reactions*, Springer-Verlag, New York.

Lauffer, M. A., 1978, Entropy-driven polymerization of proteins: Tobacco mosaic virus and other proteins of biological importance, *Dev. Biochem.* **3**:115.

Lauffer, M. A., 1980, Motion in viscous liquids: Simplified derivations of the Stokes and Einstein equations, *J. Chem. Educ.* (in press).

Lauffer, M. A., and Bendet, I. J., 1954, The hydration of viruses, *Adv. Virus Res.* **2**:241.

Lauffer, M. A., and Price, W. C., 1947, The electrophoretic purification of southern bean mosaic virus, *Arch. Biochem.* **15**:115.

Lauffer, M. A., and Shalaby, R. A., 1980, The effect of ionic strength on the entropy-driven polymerization of tobacco mosaic virus protein: Contributions of electrical work and salting-out, *Arch. Biochem. Biophys.* **201**:224.

Lauffer, M. A., and Stevens, C. L., 1968, Structure of the tobacco mosaic virus particles; polymerization of tobacco mosaic virus protein, *Adv. Virus Res.* **13**:1.

Lauffer, M. A., and Szent-Györgyi, A. G., 1955, Comments on the structure of myosin, *Arch. Biochem. Biophys.* **56**:542.

Ledingham, J. C. G., 1931, The aetiological importance of the elementary bodies in vaccinia and fowl-pox, *Lancet* **2**:525.

Leonard, B. R., Jr., Anderegg, J. W., Kaesberg, P., Schulman, S., and Beeman, W. W., 1951, On the size, shape and hydration of southern bean mosaic virus and tobacco necrosis virus in solution, *J. Chem. Phys.* **19**:793.

Leonard, B. R., Jr., Anderegg, J. W., Schulman, S., Kaesberg, P., and Beeman, W. W., 1953, An x-ray investigation of the sizes and hydration of three spherical virus macromolecules in solution, *Biochim. Biophys. Acta* **12**:499.

Levine, A. J., and Teresky, A. K., 1970, Deoxyribonucleic acid replication in simian virus 40-infected cells. II. Detection and characterization of simian virus 40 pseudovirions, *J. Virol.* **5**:451.

Loeffler, F., and Frosch, P., 1898, Berichte der Kommission zur Erforschung der Maul- und Klauenseuche bei dem Institut für Infektionskrankheiten, *Berl. Zentralbl. Bakteriol. Abt. I. Orig.* **23**:371.

LoGrippo, G. A., 1951, Partial purification of viruses with an anion exchange resin, *Proc. Soc. Exp. Biol. Med.* **74**:208.

Longsworth, L. G., 1945, The diffusion of electrolytes and macromolecules in solution: A historical survey, *Ann. N.Y. Acad. Sci.* **46**:211.

MacCallum, W. G., and Oppenheimer, E. H., 1922, Differential centrifugation: A method for the study of filterable viruses, as applied to vaccinia, *J. Am. Med. Assoc.* **78**:410.

Mandell, J. D., and Hershey, A. D., 1960, A fractionating column for analysis of nucleic acids, *Anal. Biochem.* **1**:66.

Martin, A. J. P., and Synge, R. L. M., 1941, A new form of chromatogram employing two liquid phases. I. A theory of chromatography, II. Application to the microdetermination of the higher monoamino-acids in proteins, *Biochem. J.* **35**:1358.

Martin, R. G., and Ames, V. N., 1961, A method for determining the sedimentation behavior of enzymes: Application to protein mixtures, *J. Biol. Chem.* **236**:1372.

Matthews, R. E. F., 1970, *Plant Virology*, Academic Press, New York.

McEwen, C. R., 1967, Tables for estimating sedimentation through linear concentration gradients of sucrose solution, *Anal. Biochem.* **20**:114.

Meselson, M., and Stahl, F. W., 1958, The replication of DNA in *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* **44**:671.

Meselson, M., Stahl, F. W., and Winograd, J., 1957, Equilibrium sedimentation of macromolecules in density gradients, *Proc. Natl. Acad. Sci. USA* **43**:581.

Miller, G. L., and Price, W. C., 1946, Physical and chemical studies on southern bean mosaic virus. I. Size, shape, hydration and elementary composition, *Arch. Biochem.* **10**:467.

Mueller, H., 1943, The theory of electrophoretic migration, in: *Proteins, Amino Acids, and Peptides* (E. J. Cohn and J. T. Edsall, eds.), Chap. 25, pp. 623–643, Reinhold, New York.

Neurath, H., 1942, The investigation of proteins by diffusion measurements, *Chem. Rev.* **30**:357.

Neurath, H., and Cooper, G. R., 1940, The diffusion constant of tomato bushy stunt virus, *J. Biol. Chem.* **135**:455.

Neurath, H., and Saum, A. N., 1938, The diffusion of tobacco mosaic virus protein in aqueous solution, *J. Biol. Chem.* **126**:435.

Northrop, J. H., and Anson, M. L., 1929, A method for the determination of diffusion constants and the calculation of the radius and weight of the hemoglobin molecule, *J. Gen. Physiol.* **12**:543.

O'Farrell, P. H., 1975, High resolution two-dimensional electrophoresis of proteins, *J. Biol. Chem.* **250**:4007.

O'Farrell, P. Z., and Goodman, M. H., 1976, Resolution of simian virus 40 proteins in whole cell extracts by two-dimensional electrophoresis: Heterogeneity of the major capsid protein, *Cell* **9**:289.

Okada, Y., and Ohno, T., 1976, Plant viruses with mixed phenotypes, in: *Comprehensive Virology*, Vol. 6 (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 103–129, Plenum Press, New York.

Ouchterlony, O., 1958, Diffusion-in-gel methods for immunological analysis, in: *Progress in Allergy* (P. Kallos, ed.), pp. 1–78, Karger, New York.

Oudin, J., 1952, Specific precipitation in gels and its application to immunochemical analysis, in: *Meth. Med. Res.* **5**:335–378.

Parker, R. F., and Rivers, T. M., 1935, 1936, 1937, Immunological and chemical investigations on vaccine virus. I. Preparation of elementary bodies of vaccinia, *J. Exp. Med.* **62**:65–72. III. Response of rabbits to inactive elementary bodies of vaccinia and to virus-free extracts of vaccine virus, *J. Exp. Med.* **63**:69–94. VI. Isolation of a heat-stable, serologically active substance from tissues infected with vaccine virus, *J. Exp. Med.* **65**:243–249.

Parker, R. F., and Smythe, C. V., 1937, Immunological and chemical investigations of vaccine virus, V. Metabolic studies of elementary bodies of vaccinia, *J. Exp. Med.* **65**:109.

Pickels, E. G., 1943, Sedimentation in the angle centrifuge, *J. Gen. Physiol.* **26**:341.

Pusey, P. N., Koppel, D. E., Schaefer, D. W., Camerini-Otero, R. D., and Koenig, S. H., 1974, Intensity fluctuation spectroscopy of laser light scattered by solutions of spherical viruses: R17, Q, BSV, PM2 and T7. I. Light-scattering technique, *Biochemistry* **13**:952.

Qasba, P. K., Yelton, D. B., Pletsch, Q. A., and Aposhian, H. V., 1974, in: *Molecular Studies in Viral Neoplasia*, M. D. Anderson Annual Symposium on Fundamental Cancer Research, 1972, pp. 169–189, Williams and Wilkins, Baltimore.

Reed, W., 1901, Propagation of yellow fever; Observations based on recent research, *Med. Rec.* **60**:201–209. See also *Yellow Fever: A Compilation of Various Publications*, 1911, U.S. Congr., 3rd Sess., Senate Doc. No. 822, Washington, D.C.

Richards, K. E., and Williams, R. C., 1972, Assembly of tobacco mosaic virus rods *in vitro*: Elongation of partially assembled rods, *Biochemistry* **12**:4574.

Roizman, B., and Furlong, D., 1974, The replication of herpesviruses, *Comp. Virol.* **3**:229–403.

Saigo, K., 1978, Isolation of high-density mutants and identification of nonessential structural proteins in bacteriophage T5: Disassembly of L-shaped tail fibers and a secondary major head protein, *Virology* **85**:422.

Scatchard, G., 1946, Physical chemistry of protein solutions, I. Derivation of the equations for the osmotic pressure, *J. Am. Chem. Soc.* **68**:2315.

Schachman, H. K., 1959, *Ultracentrifugation in Biochemistry*, Academic Press, New York.

Schantz, E. J., and Lauffer, M. A., 1962, Diffusion measurements in agar gel, *Biochemistry* **1**:658.

Schmidt, P., Kaesberg, P., and Beeman, W. W., 1954, Small-angle X-ray scattering from turnip yellow mosaic virus, *Biochim. Biophys. Acta* **14**:1.

Schramm, G., and Bergold, G. H., 1947, Über das Molekulargewicht des Tabakmosaikvirus, *Z. Naturforsch.* **2b**:108.

Shainoff, J. R., and Lauffer, M. A., 1956, Chromatographic purification of southern bean mosaic virus, *Arch. Biochem. Biophys.* **64**:315.

Shalaby, R. A. F., and Lauffer, M. A., 1980, Entropy-driven polymerization of the protein from the flavum strain of tobacco mosaic virus, *Arch. Biochem. Biophys.* (in press).

Shalaby, R. A. F., Banerjee, K., and Lauffer, M. A., 1968, Ion binding by tobacco mosaic virus and its protein, *Biochemistry* **7**:955.

Simha, R., 1940, The influence of brownian movement on the viscosity of solutions, *J. Phys. Chem.* **44**:25.

Smith, C. E., and Lauffer, M. A., 1967, Polymerization-depolymerization of tobacco mosaic virus protein, VIII. Light scattering studies, *Biochemistry* **6**:2457.

Stanley, W. M., 1935, Isolation of a crystalline protein possessing the properties of tobacco mosaic virus, *Science* **81**:644.

Stanley, W. M., 1944, An elevation of methods for the concentration and purification of influenza virus, *J. Exp. Med.* **79**:255.

Stevens, C. L., 1975, Equilibrium ultracentrifugation of polymers: A new method of data reduction with an application to TMV protein, *FEBS Lett.* **56**:12.

Svedberg, T., and Pedersen, K. O., 1940, *The Ultracentrifuge*, Clarendon Press, Oxford.

Tanford, C., 1961, *Physical Chemistry of Macromolecules*, pp. 324–336, Wiley, New York.

Thomas, M., and Davis, R. W., 1975, Studies on the cleavage of bacteriophage lambda DNA with *Eco*RI restriction endonuclease, *J. Mol. Biol.* **91**:315.

Tsugita, A., Gish, D. T., Young, J., Fraenkel-Conrat, H., Knight, C. A., and Stanley, W. M., 1960, The complete amino acid sequence of the proteins of tobacco mosaic virus, *Proc. Natl. Acad. Sci. USA* **46**:1463.

van Holde, K. E., and Baldwin, R. L., 1958, Rapid attainment of sedimentation equilibrium, *J. Phys. Chem.* **62**:734.

van Holde, K. E., Rosetti, G. P., and Dyson, R. D., 1969, Sedimentation equilibrium of low molecular weight associating solutes, *Ann. N.Y. Acad. Sci.* **164**:279.

Wada, A., Ford, N. C., Jr., and Karasz, F. E., 1971, Rotational diffusion of tobacco mosaic virus, *J. Chem. Phys.* **55**:1798.

Williams, C. A., Jr., and Grabar, P., 1955, Immuno electrophoretic studies on serum proteins, I. The antigens of human serum, *J. Immunol.* **74**:158.

Williams, R. C., and Fisher, H. W., 1974, *An Electron Micrographic Atlas of Viruses*, C Thomas, Springfield, Ill.

Winocour, E., 1968, Further studies on the incorporation of cell DNA into polyoma-related particles, *Virology* **34**:571.

Wyckoff, R. W. G., Biscoe, J., and Stanley, W. M., 1937, An ultracentrifugal analysis of the crystal in virus proteins isolated from plants diseased with different strains of tobacco mosaic virus, *J. Biol. Chem.* **117**:57.

Zeiger, R. S., Salomon, R., Dingman, C. W., and Peacock, A. C., 1972, Role of base composition in the electrophoresis of microbial and crab DNA on polyacrylamide gels, *Nature* (*London*) *New Biol.* **238**:65.

Zimm, B. H., 1948, Apparatus and methods for measurement and interpretation of the angular variation of light scattering; Preliminary results on polystyrene solutions, *J. Chem. Phys.* **16**:1099.

CHAPTER 2

Use of Electron Microscopy in Virology

Harold W. Fisher

Department of Biochemistry and Biophysics
University of Rhode Island
Kingston, Rhode Island 02881

1. INTRODUCTION

1.1. Historical Background

It is now 40 years since the first electron micrographs of tobacco mosaic virus were published (Kausche *et al.*, 1939). The affection which virologists have had for the electron microscope and electron microscopists for virus specimens since that time has been extremely fruitful. As evidence of the anticipated productivity of electron microscopy, consider the following quotation from p. 274 of the second edition of the book by Burton and Kohl (1946):

> Stanley and Anderson state as their considered opinion: "Although excellent micrographs of bacteria have been obtained by means of this apparatus and have proved of value in supplementing information already available, it would appear that the electron microscope will be of greatest value in the microscopy of objects having sizes between 5 and 250 mμ, a range not covered by the light microscope and one in which practically all viruses have been found to fall. The electron microscope offers the possibility of securing micrographs of individual virus particles and thus of establishing their sizes and shapes with some precision. It should also be possible to determine the extent of the variation in the size and shape of a given virus and even perhaps learn something of the mechanism by means

of which a virus particle is duplicated within the host and of the nature of the difference between strains of a given virus."

This chapter will attempt to examine how this prediction by Stanley and Anderson is progressing. First, it will survey the methods of specimen preparation that have been developed and then it will consider some of the results that have been obtained with virus specimens. Justice cannot be given to the burgeoning literature on this topic; thus the chapter will be somewhat biased by my limited familiarity with classical and current references. For a complete presentation on principles and techniques of electron microscopy, the reader should consult the series beginning in 1970 edited by M. A. Hayat (1970).

As one last note of background, I would like to point out that electron microscopy is a visual art. To quote Professor Robley Williams speaking to my 1947 class at the Harrison M. Randall laboratory, University of Michigan, "What is one microscopist's reality may be another's artifact."

1.2. The Microscope

Many improvements have been made in electron microscopes since the time of the book by Burton and Kohl (1946). Nevertheless, an electron microscope is neither portable nor inexpensive, and, to quote from the preface to the first edition of that book, "we still have the feeling that it is ever 'a dear season for cucumbers.'" The price of a good instrument still remains equivalent to the price of a moderately expensive house.

For the routine operation of the modern electron microscope, there are few of the old, time-consuming problems that hampered workers at the time of the book by Burton and Kohl (1946). The old problems of vacuum seals and electron tubes are now considered mundane and have been replaced by topics such as resolution, contamination, beam damage, thermal drift, and photography (which has always been an integral part of electron microscopy).

For instance, the development of a contamination layer on the surfaces (including the specimen) exposed to the electron beam has been known for some time (Watson, 1947). This contamination layer reduces the contrast (and resolution) of the exposed area at a significant rate. The layer is caused by the condensation and polymerization by the electron beam of hydrocarbon molecules which are residual in the

vacuum system from grease, gaskets, pump oils, and so forth. Anticontamination devices have been designed (Heide, 1965) in which the specimen is surrounded by a cold trap to reduce the partial pressure of the gases near the specimen. Such accessories are part of current microscopes.

The damaging effect of the electron beam itself has also been demonstrated for the finest details of structure of particles as small as viruses (Williams and Fisher, 1970; Thach and Thach, 1971). Fortunately, techniques have been devised to minimize this. One technique, called minimal beam exposure, has been developed wherein the specimen is subjected only to the irradiation necessary to obtain a photographic exposure (Williams and Fisher, 1970). The improved appearance of negatively stained virus particles when micrographed by this technique has been documented (Williams and Fisher, 1974). A thorough review of studies of specimen damage in the electron microscope has been published by Isaacson (1977).

2. SPECIMEN METHODS

2.1. Support Films, Shadowing, and Staining

Most of the successful methods for the preparation of specimens on electron microscope grids have depended in large part on simple and fast methods for the preparation of support films and reliable methods for contrast enhancement. The support films had to be strong, thin, relatively structureless, and clean. The contrast of a specimen, such as a virus particle supported by a film of more than 5 times its thickness, had to be increased by techniques which would not distort its characteristics. It is not surprising that many textbooks, review articles, and workshops have given considerable attention to water purity, support film preparation, clean glassware, and specimen techniques which avoid detergents, nucleases, oils, and salts (Evenson, 1977; Hayat, 1972; Kay, 1965).

The main kinds of support films which have been in general use are plastic (Parlodion or Formvar), carbon, and carbon-coated plastic. The advantages and disadvantages of each are as follows. Water-cast Parlodion has been described as the easiest and fastest to prepare (Kay, 1965). It gives a good hydrophilic surface but has the disadvantage of "dimensional instability" under the beam in the electron microscope. Formvar films have been prepared either by dropping on water (water-

cast) or by dipping a clean glass slide into a dilute solution of 0.3% Forvar in 1,2-dichloroethane and, after drying, floating the film on water. Pure carbon films are even stronger than Formvar. They have the finest structure and can be made as thin as 0.5 nm (Williams and Glaeser, 1972). However, carbon films are fragile, not easily stuck to the grid, and not readily wettable. A popular compromise has been a support which is a combination of Formvar film and carbon coating made hydrophilic by a glow discharge for about 5 sec.

Both shadowing and staining methods have been used to increase the contrast by decorating the specimen with heavy metal but with minimal increase in background "noise." Shadow casting was one of the earliest techniques (Williams and Wyckoff, 1945) applied to virus particles and to crystalline plant viruses (Price *et al.*, 1945). The method was originally devised to measure the height of objects (Williams and Wycoff, 1944), and subsequently was used to demonstrate features such as polyhedral form (Williams and Smith, 1958; Bils and Hall, 1962) by shadowing at two angles. For viral nucleic acids, the method has been modified to rotary shadowing at a small grazing angle (Heinmets, 1949; Kleinschmidt, 1968). Recent studies have been carried out with tungsten shadowing since metal deposition seems to have a greater potential for increased resolution than current methods for staining nucleic acids (Griffith, 1973; Hart, 1963; Abermann *et al.*, 1972).

Staining the specimen has the advantage of speed and simplicity and the potential for specificity. The staining techniques have been either "positive" or "negative" depending on whether the virus particles or their proteins and nucleic acids have bound the heavy metal (as discussed in Section 2.2) or whether the virus particles are embedded in the heavy metal (as discussed in Section 2.3). Osmium tetroxide, lead citrate, and uranyl salts have been used extensively in positive staining, and phosphotungstate and uranyl salts have been used for negative staining.

2.2. Cytological Techniques

Studies of virus infection, virus release, and virus-infected cells have involved all of the standard cytological techniques for electron microscopy which evolved over the years from the traditional paraffin wax methods used for light microscopy. These methods include fixation, dehydration, embedding, staining, thin sectioning, special tech-

niques for tissue culture cells, vertical sections, and specific labeling. For in-depth coverage of the methods, one can consult any of a large number of source books (see, for instance, Dawes, 1971; Glauert, 1972; Hayat, 1972; Pease, 1964; Sjostrand, 1967).

It is rather amazing that an unusual compound, osmium tetroxide, has been found to be a good fixative for histological purposes for over a hundred years, and also to yield good results in fixation for electron microscopy of some of the earliest applications to cells and tissues (Porter *et al.*, 1945; Porter and Kallman, 1953). There have been many descriptions of the use of fixatives such as acrolein (Luft, 1959), formalin (Holt and Hicks, 1961), glutaraldehyde (Sabatini *et al.*, 1963), and permanganate (Luft, 1956), but none of those studies has been particularly concerned with the electron microscopy of virus-infected cells. Generally, the preservation of cell membranes and organelles has been used as a criterion for the determination of the effectiveness of the fixation. In particular, the unit membrane (Robertson, 1964) was widely used for the evaluation of the preservation of the specimen.

Since the classical systematic study by Palade (1952), the importance of buffering during fixation has been emphasized. An example of a widely used procedure has been a prefixation in a dilute Karnovsky's (1965) fixative (1% paraformaldehyde, 3% glutaraldehyde) in 0.1 M sodium cacodylate buffer (this arsenic-containing buffer is an enzyme poison) at pH 7.4 for 2 hr at room temperature. The sample was then postfixed in 1% OsO_4 and dehydrated in ethanol since final embedding has most frequently been in nonaqueous polymers.

A most important improvement in embedding over the earliest work was the use of plastics rather than paraffin (Newman *et al.*, 1949). In that study, methacrylate as a monomer was infiltrated into the dehydrated tissue and then, with the aid of an initiator, polymerized. Although methacrylate was used for about 10 years, it was eventually replaced by other polymers which caused fewer problems with shrinkage and artifacts. Two examples of the improved epoxy-type resins are Araldite (Glauert *et al.*, 1956) and Epon 812 (Luft, 1961). Later, mixtures of these two were also found effective (Mollenhauer, 1964).

Two major advances in the development of ultramicrotomy were made in the cutting machine and the knife. Three basic modifications of the standard microtome advance mechanism were made by Porter and Blum (1953), Sjostrand (1953), and Huxley (1958) to produce the required thinness.

Latta and Hartmann (1950) replaced the steel knife by a glass knife which produced an edge capable of cutting the plastic block into

ultrathin sections. The diamond knife was introduced by Fernandez-Moran (1953), but took many years of development before it became consistently sharp and relatively trouble-free on the commercial market.

There have been several developments in cytological techniques which have been interesting and useful for virologists. For example, virus-infected cultures have posed the problem of *in situ* fixation and embedding in order to preserve the spatial arrangement existing during virus growth and development. One approach taken by a number of investigators studying the ultrastructure of cell attachment to supporting surfaces has been to grow the cells directly on a plastic compatible with the embedding polymer, such as Epon disks (Zagury *et al.*, 1968), Araldite disks (Abercrombie *et al.*, 1971), or Millipore filters (McCombs *et al.*, 1968). Other workers have developed techniques which can be used with the tissue culture vessel itself (Brinkley *et al.*, 1967; Douglas and Elser, 1972). Many of the methods have been designed to permit vertical sectioning of the tissue culture cells (Fisher and Cooper, 1967; Dalen and Nevalainen, 1968; Nelson and Flaxman, 1972).

Another area of development of specimen techniques of particular value to virologists has been the specific tagging of viruses and antibodies with radioisotopes and immunoferritin. The reasons that radioautography has been an attractive tool are as follows: (1) it allows the specific localization of the virus or virus material within the cell; (2) as in a pulse-chase experiment, it can permit the designation of a sequence of events within some time frame; and (3) by controlling the amount of radioisotope, it is possible to do some quantitative determinations. Most of the contemporary methods began with the studies of Caro (1962), Caro and Van Tuburgen (1962), and Salpeter and Bachmann (1964) on the problems of high-resolution autoradiography and the special difficulties in the application of it to electron microscopy. The basic techniques were thoroughly reviewed [see Salpeter and Bachmann (1972), Salpeter (1966); Stevens (1966) for a very popular loop method]. One interesting variation of the standard techniques has been the application of autoradiographic methods to particulate isolates which have been stained or shadowcast (Maraldi, 1976). Prior staining of free particles has given higher resolution because the heavy metal stain confines the radioactive emission to the immediate vicinity of the particles. Also, a specimen of particles need not be as thick as an embedded section. Other interesting methods have been the localization of intracellular DNA by binding radioactive actinomycin (Geuskens, 1974) and the localization of vegetative viral DNA replication by

hybridization with complementary radioactive RNA (Croissant *et al.*, 1972; Geuskens and May, 1974).

One of the advantages of immunochemical techniques has been the simple specimen technique, since the electron-dense ferritin is readily visible in the microscope (Singer, 1961). Since it has been possible to use an indirect immunoferritin technique (Levinthal *et al.*, 1967), there have been a variety of specific proteins available for tagging. One of the main disadvantages of these methods has been the failure to obtain penetration of the antibody into the sections. All tagging is on the surface of embedded sections. Another problem has been the background of nonspecific ferritin which adheres to the surface.

The use of colloidal gold as an electron-dense marker has been claimed to have several advantages over peroxidase and ferritin conjugates (Yeger and Kalnins, 1978). Gold-labeled antibodies are relatively easy to prepare, can be separated from free antibody by centrifugation, and can easily be seen in the electron microscope because of their density. Immunolatex spheres (Gonda *et al.*, 1978) have been compared to other electron-dense markers for cell and virion surface labeling because their distinct shape and size also allows identification in the scanning electron microscope.

2.3. Negative Staining

One of the techniques designed to overcome the problems of contrast was negative staining or heavy metal embedding. The idea was first discussed by Hall (1955) in a study of positive staining and subsequently very elegantly demonstrated by Brenner and Horne (1959) as a method to aid high-resolution electron microscopy of viruses. It has gained wide popularity because of its simplicity and the lack of a requirement for expensive or complicated equipment. Indeed, as we shall see in Section 3.2, it has paid off handsomely in the study of viruses. Nevertheless there have been some problems of technique and interpretation.

Contrast has always been a major problem for electron microscopy. What one means by contrast is the difference of intensity between adjacent points in the image or the variations of photographic density of nearby regions in an electron micrograph. These variations are the results of the difference in elastic scattering of the electron beam by the specimen material. What one would like to know is what property of the stain material will give maximal difference in intensity

and thus contrast. This has been worked out theoretically (for greater detail, see Hall, 1966, Chapter 8). An expression for this is given by

$$\frac{I}{I_0} = e^{-S_c \rho \Delta x}$$

where I/I_0 is the ratio of the transmitted to the incident intensity, S_c is a scattering constant which includes the geometry and all factors independent of the chemical composition, and $\rho \Delta x$ is the mass thickness (of the stain). A study of a number of substances suitable for use as a negative stain based on this mass thickness was undertaken by Valentine and Horne (1962) using polystyrene latex spheres as a specimen object. Their results are reproduced in Table 1.

From a practical point of view it has been clear that some technical problems have not been solved. For high-resolution work, the advantage of not requiring high-purity samples for negative staining becomes a disadvantage if bovine serum albumin or a virus protein must be added to obtain good spreading of the specimen. Characteristics such as wettability, pH, ionic strength, and purity of a commercial supply of a stain may be very important in good negative staining (Williams and Fisher, 1974; Hirsh and Schleif, 1976). At this writing, it is still impossible to know which of the most popular stains (uranyl acetate, uranyl formate, uranyl oxalate, phosphotungstic acid, etc.) will consistently give the best result (Haschemeyer and Myers, 1972).

Problems of interpretation of micrographs of negatively stained virions arise from the superposition of the protuberances on both the

TABLE 1

The Relation between Negative Contrast and the Anhydrous Density of a Selection of Substances Tried for Negative Staining Purposes[a]

Chemical	Anhydrous density ($g\text{-}cm^{-3}$)	Contrast between stain and polystyrene spheres
Thalium carbonate	7.1	0.80
Cadmium	5.7	0.55
Silver nitrate	4.4	0.35
Sodium tungstate	4.2	0.37
Uranium nitrate	3.7	0.29
Sodium dihydrogen phosphate	2.4	0.11

[a] From Valentine and Horne (1962).

bottom and the top of a virus particle in the same image (Klug and Finch, 1968; Williams and Fisher, 1974). Computer-simulated images (Klug and Finch, 1968) and optically filtered one-side images (Kiselev and Klug, 1969) of virus particles have been used as aids in arriving at models for the structure of some viruses. A negative-staining carbon film technique for studying viruses in the electron microscope has been devised by Horne and co-workers (Horne and Ronchetti, 1974; Horne *et al.*, 1975). A chapter on the structure of small DNA-containing animal viruses which examines these methods thoroughly can be found in Volume 5 of this series (Finch and Crawford, 1975). Markham *et al.* (1963) also proposed a photographic method to examine symmetry of virus particles in cases where the images are equivocal. The caution which should be used with this technique has been described (Friedman, 1970).

An unanticipated but enormous benefit of the negative staining technique has been its relatively good preservation of three-dimensional structure of virus particles during drying. Most of the viruses are minimally flattened or distorted as drying proceeds, presumably because a matrix of stain in the form of a glass fills the spaces formerly occupied by the solvent. Nevertheless, it has been adequately demonstrated by Nermut (1972) that freeze-dry negative stain will prevent pleomorphism of some viruses caused by air drying. It also has been recognized that exposure to the electron beam in traditional electron microscopy may be quite damaging to the finest details of a virus even in the matrix of a negative stain (Williams and Fisher, 1970).

2.4. Spreading Techniques (with and without Monofilms)

A number of ingenious techniques have been devised for the examination of viral nucleic acids and nucleic acid complexes with proteins. Most of these methods began with the elegant technique described by Kleinschmidt and Zahn (1959). The methods have been modified over the years and have become very reliable and routine for nucleic acid characterization and genetic mapping (see Section 3.3). More recently, with the exact determinations made by restriction enzymes and rapid DNA sequencing methods, there has been considerable activity in the electron microscopic studies of replication, recombination, transcription, and translation. The techniques perfected for the electron microscopic visualization of nucleic acids and their complexes with proteins were recently reviewed by Fisher and Williams (1979).

In general, the purpose of the techniques has aimed at the conver-

sion of the nucleic acid fibers from their natural three-dimensional arrangement in solution into a dry, two-dimensional array for electron microscopy. Ideally, the purpose was to produce no entanglement or shear and as little distortion of the conformation and interbase spacing as possible. In addition, the recent methods have been aimed at the highest resolution with the least obscuring of the specimen strands and bound proteins.

A schematic representation of several of the methods in which surface films have and have not been used are illustrated in Fig. 1. The classical method of Kleinschmidt is shown at the top (Fig. 1A). In this method, the nucleic acid in solution was transferred to a protein monolayer by adsorption to the basic groups of the surface-denatured film. This film was presumed to be a network of unfolded peptide chains, and the basic side groups of some of the amino acids were the attachment sites for the negatively charged phosphate groups of the nucleic acid backbone. In this way, the nucleic acid molecules were fixed in an insoluble stable protein film prior to transfer onto the specimen grid. In Fig. 1, step A shows the introduction of the spreading solution (hyperphase) onto the hypophase in a trough. This was done by allowing a small volume (50–100 μl) of the spreading solution to run down a clean glass slide which has been previously wetted with the hypophase. The trough was made hydrophobic and the surface of the hypophase swept clean using a clean hydrophobic bar. The bar was left in place at the distal end of the trough for the next step. Normally, the spreading solution was made with the nucleic acid at 1–5 μg/ml in 0.4–4 M ammonium acetate buffer at pH 8 and cytochrome *c* at 100 μg/ml as the spreading protein (Kleinschmidt, 1968). Other proteins such as trypsin and lysozyme have not produced more consistent success than that achieved with cytochrome *c*. The hypophase usually contains about 0.15 M ammonium acetate buffer, but variations of this have been used to produce lysis or release of nucleic acid from some specimens.

Step B in Fig. 1A shows how workers have slightly compressed the protein monolayer with the bar just before they touched the previously prepared grid to the surface. A few flecks of talc have sometimes been sprinkled on the surface to detect the formation of the film as it comes off the glass slide and also to observe the compression.

A modification of this method, which also uses a cytochrome *c* surface film, was devised by Lang *et al.* (1964). This "diffusion method" is illustrated in the middle of Fig. 1B. This technique has been advocated because of the possible enhanced control of the ionic envi-

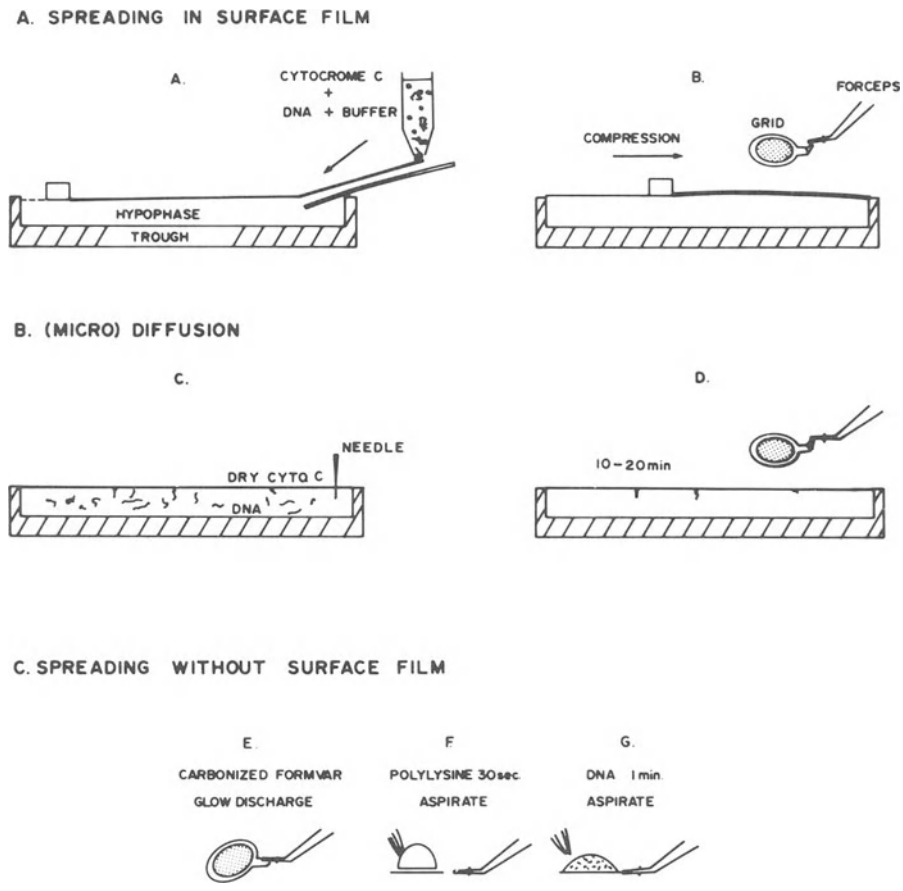


Fig. 1. Methods of spreading viral nucleic acids.

ronment and transport of the nucleic acid to the film. Also, it was subsequently modified to a microdiffusion method (Lang, 1972). In step C a needle with dry cytochrome *c* is shown to prepare a film over a solution of 0.2 M ammonium acetate containing as little as 5×10^{-8} μg/ml of DNA. After a 10–20 min wait (step D), the DNA was found to have diffused to the surface film and irreversibly adsorbed to the lower surface. The specimen grid was then touched to the surface, washed, blotted, and stained or shadow-cast.

A significant alteration of the buffer system was introduced by Westmoreland *et al.* (1969). She exchanged the usual aqueous volatile ammonium acetate for a formamide procedure, which found extensive use for mapping and heteroduplex analysis. These methods, which were

reviewed by Davis *et al.* (1971), used spreading solutions containing about 0.5 μg/ml of DNA, 0.02–0.1 mg/ml of cytochrome *c*, and Tris and EDTA buffered to pH 8.5 and 40% formamide. The hypophase was made to 10% formamide, also with Tris and EDTA at pH 8.5, immediately before use since these solutions become acidic rather rapidly.

Several attempts have been made to replace the cytochrome *c*, a protein of almost 10 nm diameter, which is bound to the DNA and hence obscures details. For instance, Vollenweider *et al.* (1975) used a compound of much lower molecular weight called benzyldimethylalkylammonium chloride (BAC) in the spreading solution at a final concentration of 0.0025% with the buffer and nucleic acid. This method was applied by Evenson (1977) but with variable results, and it has not yet found widespread use.

Several other methods for displaying nucleic acid without obscuring surface films have been devised. One of these techniques (Koller *et al.*, 1974) used ethidium bromide, a trypanocidal drug that has been used as a stain for nucleic acids in gel electrophoresis. This compound has long been known to bind with double-stranded polynucleotides as an intercalated complex. This complexing obviously distorts the DNA structure and is not applicable to single-stranded nucleic acids. The ethidium bromide method was used by Arnberg and Arwert (1976) to locate in the electron microscope the position of a protein which presumably acted as a linker in the circularization of *Bacillus* bacteriophage GA-1 DNA.

It has been shown that carbon films subjected to ionic bombardment in a glow discharge are modified so that they will bind nucleic acids (Reissig and Orrell, 1970; Highton and Whitfield, 1974; Griffith, 1973). The treatment was found not to be permanent and the films produced showed variable results depending on the discharge time and current. Glow discharge in a partial atmosphere of amylamine has also been shown to bind nucleic acids and avoid the aggregation frequently produced on drying (Dubochet *et al.*, 1971).

An attractive method was recently described by Williams (1977) for spreading without surface films. It is illustrated schematically at the bottom of Fig. 1. His method used poly-L-lysine, which was known to bind DNA molecules. It was carried out with a procedure which required very little sample. He used electron microscope specimen grids of the handle type filmed with Formvar and coated with a thin layer of carbon (step E). The films were then made hydrophilic by subjecting them to a glow discharge for about 15 sec. To prevent the spread of

liquid to the tips of the forceps used to hold the grid, a small piece of metal was placed over the handle during the glow discharge, thereby keeping the handle hydrophobic. In step F a small drop of 5–8 μl of polylysine at 0.3–1.0 μg/ml in water was placed on the grid for 30 sec and quickly aspirated with a flame-drawn pasteur pipet. The successful preparation of the surface was monitored by inspecting the drying of the residual liquid under a 10-power microscope. A hydrophilic surface was found to take several seconds to dry. In the step G the nucleic acid was adsorbed for about 1 min at a concentration of 0.1–2.0 μg/ml in buffer and then aspirated away. The buffer required Mg^{2+} at greater than 3 mM, and auxilliary substances did not interfere as long as they were readily soluble in water. (Binary complexes of DNA and RNA polymerase were adsorbed in a manner identical to that described for DNA alone.) Satisfactory concentrations of RNA polymerase ranged from 1 μg/ml with 3-min adsorption time to 10 μg/ml for 20 sec. For rinsing, drops of pure water were lined up on a freshly scraped teflon sheet and the inverted grid was touched to each drop without allowing it to dry until the final rinse.

2.5. Freeze-Drying, Freeze-Etching, and Related Techniques

The importance of techinques designed to avoid the distortions of structures associated with drying has been recognized for a long time. Two methods were developed around 1950 for preventing these artifacts in the preparation of specimens for electron microscopy. Both of these methods were designed to avoid the crossing of the specimen by a liquid–vapor phase boundary with its accompanying high surface tension. In one method, freeze-drying, the specimen was frozen and dried out of an ice matrix at low temperature by vacuum sublimation (see, for instance, Williams (1953*a*). The other method, critical-point drying, was developed by Anderson (1951) in an elegant demonstration of bacteriophage attachment to host cells. In this ingenious technique, the specimen was transferred from a water solution to another solvent miscible in both water and liquid carbon dioxide. The specimen was then placed in a bomb and flushed with liquid carbon dioxide at high pressure and room temperature. When the bomb was completely filled, the temperature was raised to 35°C, a temperature above the critical point of carbon dioxide, and the liquid was imperceptibly converted to a gas without a liquid–gas interface. At this slightly elevated temperature, the pressure was then reduced and the specimen dried from a gaseous

environment. Carbon dioxide was chosen because the critical temperature is not too high and hence not damaging to biological specimens. The critical-point drying method has been found very practical for cells and tissues for scanning electron microscopy (Cohen, 1974). A complete review of freeze-drying for electron microscopy was made by Nermut (1977).

In the first freeze-etch studies of virus crystals, Steere (1957) assumed that the fracture surface of a frozen sample cleaved with a cooled scalpel would reveal increased detail by controlled sublimation of surface ice. Since those studies, there has been considerable refinement of the techniques and increased reliability of the equipment, leading to a new and sophisticated technology for examining internal structures, organelles, membranes, and virus-infected cells.

The general procedures for freeze-etching are shown in Fig. 2. In the first step, the freezing of the sample is illustrated. Although one can pretreat the specimens before freezing with cryoprotectants or glutaraldehyde fixation, a great advantage of the freeze-etch method is the possibility of seeing ultrastructures without the artifacts of those treatments, or at least with different kinds of artifacts, such as intracellular ice crystals. Cryoprotective substances, which have been examined at a variety of concentrations, are ethylene glycol, glycerol, and dimethyl sulfoxide. These have been shown to be important in affecting the freezing rate. In the usual procedure, in step 1, the specimen has normally been frozen in liquid Freon 22, cooled in liquid nitrogen almost to $-165°C$, the freezing point of the Freon. The specimen can then be transferred to liquid nitrogen and then to the freeze-etch unit (several commercially available units are in current use; see Koehler, 1972). In step 2, with the temperature at $-185°C$, a scalpel or knife is manipulated to achieve the fracture in the vacuum chamber. After fracturing, the two cleaved surfaces are then etched in the vacuum at 1×10^{-5} torr at $-100°C$. The control of temperature and pressure is very important in step 3. During etching, the specimen faces need protection against contamination by condensation of hydrocarbon vapors within the vacuum system. Several methods have been described to do this (Moor *et al.*, 1961; Steere, 1969). In step 4, a replica of the surface is prepared, still at $-150°C$ and 1×10^{-5} torr, by shadowing with platinum wire wrapped around a carbon point, followed by a support coating of carbon film. In step 5, the replica is removed from the vacuum chamber and cleaned in dilute chromic acid (plant material) or dilute sodium hypochlorite (animal tissue). Finally, in step 6 the replica is rinsed in distilled water and mounted on an electron microscope grid for viewing.

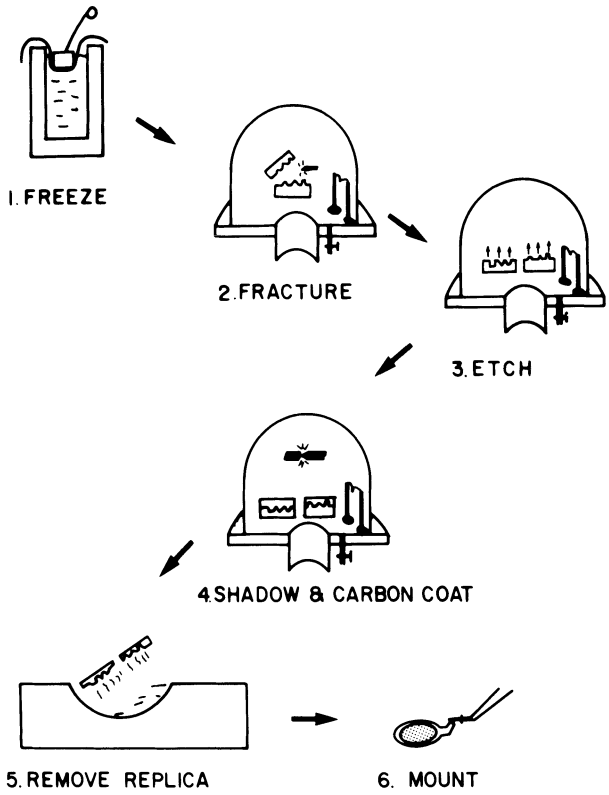


Fig. 2. Schematic representation of the method of freeze-etching.

The replica techniques illustrated in Fig. 2, steps 4, 5, and 6, were developed over a number of years originally for analysis of surface details of electron-opaque materials such as diamonds, metals, and glass. The technique of shadow-casting (Williams and Wyckoff, 1946) stimulated a variety of ways to produce surface replicas (Hall, 1966), and the development of carbon evaporation as a stabilizing film (Bradley, 1954) brought widespread use and eventually significantly high resolution (Henderson and Griffiths, 1972).

The accompanying electron micrograph of a freeze-etch preparation shown in Fig. 3 illustrates the value of the technique. In the complete study by Steere and Erbe (1979), complementary areas opening like a book were presented as stereo pairs. Their comparison showed the difference between freeze-fracture and freeze-etch specimens of potato yellow dwarf virus in the space between the two nuclear membranes. In the freeze-etched portion, shown here, the spikes (peplomers) were clearly revealed, whereas the freeze-fractured complement provided very little information of this sort.

Fig. 3. Replica of a freeze-etch preparation of unfixed *Nicotiana rustica* cells infected with potato yellow dwarf virus (no cryoprotectant added). Magnification ×75,000 (reduced 38% for reproduction). Courtesy of R. L. Steere.

The micrographs of freeze-etch materials can be confusing, and a bewildering assortment of nomenclatures has been in use to describe and label these fracture faces and surfaces. To reduce confusion, Steere customarily prepares intermediate photographic negatives from the original electron micrographs and then makes prints (as shown in Fig. 3) with black shadows pointing down or to one side. A system for nomenclature has been proposed (Branton *et al.*, 1975) which generates a mnemonic labeling scheme that relates topologically equivalent portions of different membranes.

2.6. Measuring and Counting Virus Particles and Components

The electron microscope does more than provide a visual presentation to be used in the attempt to correlate structure and function. It is also a tool with which one can measure size and count the numbers of each species observed, both within tolerable limits of error.

The calibration of the magnification of the microscope has been most frequently done with a replica of a diffraction grating or with indicator particles included with the specimen or applied to the reverse side of the film. One of the most widely used indicators has been the small, spherical particles of exceptionally uniform size of polystyrene latex (PSL) first described by Backus and Williams (1949). The possible errors introduced by shrinkage, contamination, and other sources have been discussed by many workers (Hall, 1966). Frequently, a biological specimen has been used for internal size calibration. For example, tomato bushy stunt virus (BSV) and tobacco mosaic virus (TMV), which are well characterized viruses, have been used as size indicator particles (Kirschner *et al.*, 1975; Sjostrand, 1967). ϕX174 RF DNA, illustrated in Fig. 5, has been used as a length standard for linear measurements of strands of nucleic acid in monofilms (Fisher and Williams, 1979). The contour length of the unknown nucleic acid strand can be obtained by a comparison of the measurements from a linear integrator of the standard on the micrograph.

Counting virus particles and viral components was another early quantitative application of electron microscopy. There were three major methods developed for such counting: (1) the spray drop, (2) sedimentation, and (3) agar filtration, and a number of adaptations of these methods. The spray drop counting method was an outgrowth of the discovery of very uniform polystyrene latex spheres (Backus and Williams, 1949) and the development of suitable volatile buffers (Backus and

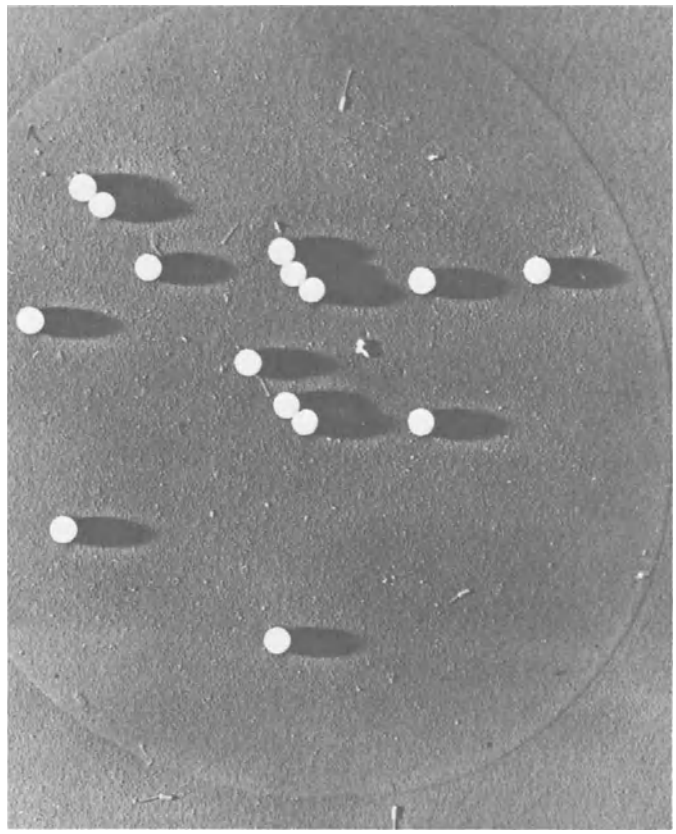


Fig. 4. Spray drop pattern of an unknown sample of bacteriophage of *Bacillus subtilus* containing polystyrene latex spheres for quantitation. Magnification ×20,000 (reduced 50% for reproduction).

Williams, 1950) (see Fig. 4). Adaptations of the spray drop method include freeze-drying (Williams, 1953*a*) and negative staining (Watson, 1962).

The sedimentation method followed a proposal by Crane (1944) for direct deposition of specimen materials onto electron microscope specimen films and was extensively used by Sharpe (1949), Sharpe and Beard (1952), Sharpe and Buckingham (1956), and Sharpe and Overman (1958). The use of agar as the cushion for sedimentation (Sharpe and Beard, 1952) demonstrated the advantage of agar for the removal of salts. Kellenberger and Arber (1957) made use of this filtration characteristic of agar for quantiative analysis of phage lysates. All

these methods have been extensively reviewed (Miller, 1974; Sharpe, 1965; Isaacs, 1957).

3. SURVEY OF RESULTS

3.1. General Characterization of Virus Particles

The size and shape (morphology) determined by electron microscopy have for long been the simplest and most direct method for the physical characterization of the etiological agent of a virus disease. Even when the direct visualization regarding virus morphology only portrayed the structures as rods, spheres, bricklike, or tadpole shape, it settled controversies of long standing and pointed out new problems (Delbrück, 1946). This appealing ability of the electron microscope to convey an immediate visual "reality" to a newly isolated virus has made it one of the first and foremost tools of identification that investigators have at their disposal. Some of the earlier techniques for the electron microscopy of viruses (Williams, 1953*b*) have largely been replaced by negative staining methods (Nermut, 1972) which are faster, simpler, and reveal far more structural detail and hence permit a classification by architectural principles, which will be discussed below (Section 3.2).

Some of the studies which have been carried out on the electron microscopy of virus growth will be considered in Section 3.5. At this point, it should be noted that some general cytological characterization has been done to identify "crystalline virus inclusions" and their localization inside the infected cells. Examples of such localization are the crystalline arrays of herpes virus, adenovirus, and papovavirus found inside the nucleus and of poliovirus found in the cytoplasm (Bernhard, 1964). There are also the so-called factories of vaccinia virus in the cytoplasm of infected cells (Cairns, 1960; Dales, 1963).

Another example of general characterization of viruses has been the surface projections which are clear and distinct on mammary tumor viruses (see Fig. 7). These viruses have been called B-type particles. It has generally been believed that the group of leukemia viruses (the so-called C-type particles) lack these surface projections (deHarven *et al.*, 1973). Great care must be exercised in the interpretation of such preparations because viruses are of a size sensitive to surface tension (Nermut, 1977). Type A particles resemble morphologically the cores

of immature types B and C particles (Provisional Committee for Nomenclature of Viruses, 1966).

3.2. Capsid Structure and Virus Classification

The electron microscope has reigned supreme as the instrument to uncover the symmetrical elements in the architecture of virus particles. Although in an earlier study (Williams *et al.*, 1960) it was shown that the "knobs" on shadow-cast papilloma virus particles could be counted, the improved resolution obtained with the negative staining technique made it the new method of choice. This technique, described in Section 2.3, was simple, fast, and revealed regular surface structure not previously recognized on virus particles. The symmetry and regularity of the protuberances on the surface of virus particles are sometimes very striking and have now been noted on almost all electron micrographs of negatively stained preparations (Horne and Wildly, 1961; Williams and Fisher, 1974; see also the micrographs in the appendix to Volume 1 of this series). These protuberances or morphological units have been called "capsomers"; in virus architecture, they are arranged to form the protein shell, or *capsid*, of the particle. Unlike most of the objects of the biological world, virus particles have been extremely uniform in size and shape and could form crystals or paracrystals both as inclusion bodies (Bernhard, 1964) in infected cells and as purified preparations (Stanley, 1935).

The earliest indications that a set of architectural principles must underlie this uniformity and regularity came after Crick and Watson (1956) noted the limited coding capacity of viral genomes. It was speculated that the proteins of the shell must occur as multiple copies of a subunit, much in the way that bricks occur in multiple copies in a building. Caspar and Klug (1962) made the observation that, except for the tailed bacteriophage, all of the simpler viruses were either rod shaped or almost spherical. They proposed that simple principles of construction could account for this. For instance, the rods could arise from a helical assembly of repeating asymmetrical subunits, and the almost spherical particles could come from an isometric assembly.

Of course, the most familiar of the viruses with helical symmetry is tobacco mosaic virus (TMV). TMV has been intensively studied by both electron microscopy and X-ray diffraction techniques, and the structure has been described with a resolution of less than 0.7 nm (Holmes *et al.*, 1975; Stubbs *et al.*, 1977). Other examples of helical

assemblies are the more flexuous potato virus X and filamentous bacteriophage and the fatter tobacco rattle virus and bullet-shaped vesicular stomatitis virus. (See Chapter 3 for X-ray and neutron scattering.)

Viruses with isometric symmetry have required more detailed explanation. Most of these viruses were found to have icosahedral or 5 3 2 symmetry (meaning that there are 5-fold, 3-fold, and 2-fold axes of symmetry). Caspar and Klug (1962) had suggested on theoretical grounds that the capsids are built of repeating capsomers packed to comply with cubic symmetry. A modification of the original ideas, the concept of quasi-equivalence, was necessary to accommodate the distortion caused by all bonding between identical subunits fitting into planar facets.

An icosahedron is built of 20 identical equilateral triangles, with 12 vertices and 30 edges. The faces of the icosahedron, of course, could be further subdivided into smaller equilateral triangles. The triangulation number, T, is given by $T = Pf^2$, where $f = 1,2,3, \ldots$. In the case of the icosahedron, $P = 1$ and T is $1,4,9, \ldots$ and each subtriangle must contain three subunits in identical, equal-spaced array (see Williams and Fisher, 1974; Caspar and Klug, 1962; Fenner, 1968). The triangulation numbers for the virus capsids have been estimated by direct counting of capsomers on electron micrographs, but the results are not necessarily straightforward.

A better understanding of the problems of direct counting, handedness, and superposition can be found in the discussion by Finch and Crawford (1975) in Volume 5 of this series. They reviewed the question of how surface patterns are generated by subunits to form clusters in well-defined groups that are easily resolved by the electron microscope. Finch and Crawford also showed how the large morphological units seen on the surfaces of polyoma and papilloma viruses are consistent with the hexamer and pentamer clustering of units at the vertices of the surface lattice, at which are located the local 6-fold and 5-fold rotational symmetry axes.

Obviously, there are numerous possibilities for different assemblies even under the simple principles of construction proposed by Caspar and Klug (1962). An example of a possible deviation from the simple rules has been the study of chloris striate mosaic virus (Hatta and Francki, 1979). These particles have been shown to be polyhedral, measuring about 18 nm in diameter, but they occur as paired (geminate) structures. Each particle appears to be constructed of two incomplete icosahedra with $T = 1$ surface lattice, having a total of 22 capsomers.

A classification of viruses has been proposed based on the architectural arrangement of the capsids of the virions (Lwoff and Tournier, 1966). This has been found to be a useful system for a collection of electron micrographs of viruses (Williams and Fisher, 1974). This system is based on four discriminating characteristics as follows:

1. The chemical nature of the genetic material, either DNA or RNA, determining the subphyla.
2. The symmetry of the nucleocapsid of the virion: helical, cubic, or binal, determining the classes.
3. The covering of the nucleocapsid, either naked or enveloped, determining the orders.
4. The size of the nucleocapsid, either the diameter of the helix or the number of capsomers in the cubic system, determining the families.

The way these criteria have been utilized (Williams and Fisher, 1974) is shown in Table 2.

3.3. Viral Nucleic Acids

The spreading methods described in Section 2.4 have been the simplest and most direct ways to examine contour lengths, strandedness, flexibility, supercoiling, and circularity of viral nucleic acids. Some of the earliest studies with these spreading methods examined the general characteristics of the uncoated nucleic acids from purified virus particles and the purified vegetative forms of nucleic acid from infected cells (Dunnebacke and Kleinschmidt, 1967; Kleinschmidt *et al.*, 1963). Some caution must be exercised on what can be concluded about the conformation of the nucleic acid as it exists in the capsid, in the infected cell, or in solution as deduced from the images of a dried, two-dimensional specimen (see Fig. 5). A fair degree of accuracy has been obtained from contour length measurements with internal reference standards, and, during the last decade, there have been many reports on physical mapping of viral genomes by electron microscopy, on determinations of base-pair repeat distances of nucleic acids, and on studies of replication, recombination, transcription, translation, processing, and splicing of viral nucleic acids. Some of these studies will be discussed here and others will be considered more appropriately in Section 3.5.

The absolute value of the interbase spacing has been considered a useful property to determine on DNA spread in an electron microscope specimen. Several reports of such measurements have been reviewed

TABLE 2

Criteria for Classification of Viruses[a]

Nucleic acid	Symmetry	Presence of envelope	Size	Triangulation No.	Examples
RNA	Helical	No	Small	—	Potato virus X
			Medium	—	Tobacco mosaic virus
			Large	—	Tobacco rattle virus
		Yes	Medium	—	Influenza virus, Sendai virus, vesicular stomatitis
	Cubic	No	Small	3	Phage MS2, turnip yellow mosaic virus, satellite tobacco necrosis virus, brome mosaic virus
			Medium	3	Poliovirus, tomato bushy stunt virus
			Large	9	Reovirus, wound tumor virus, cytoplasmic polyhedrosis virus
		Yes	Medium	3	Rous sarcoma virus, Sindbis virus
DNA	Helical	No	Small		fd phage, M13
		Yes	Large		Vaccinia virus
	Cubic	No	Small	1	Phage ϕX174, G4
			Medium	7	Papilloma virus, simiam virus 40, cauliflower mosaic virus
			Large	25	Adenovirus
			Huge	147	Tipula iridescent virus
		Yes	Large	16	Herpesvirus
	Cubic, tailed (binal)	Virulent Temperate			Phage T4, phage T5, phage T7 phage λ, phages P2, P1

[a] Adapted from Williams and Fisher (1974).

(Fisher and Williams, 1979). Significant errors can arise in the determination of the base-pair repeat distance, particularly in any comparison with completely different methods of evaluation such as X-ray diffraction analysis of paracrystals of nucleic acid. Sources of error which have been noted in the use of the spreading method have been discrep-

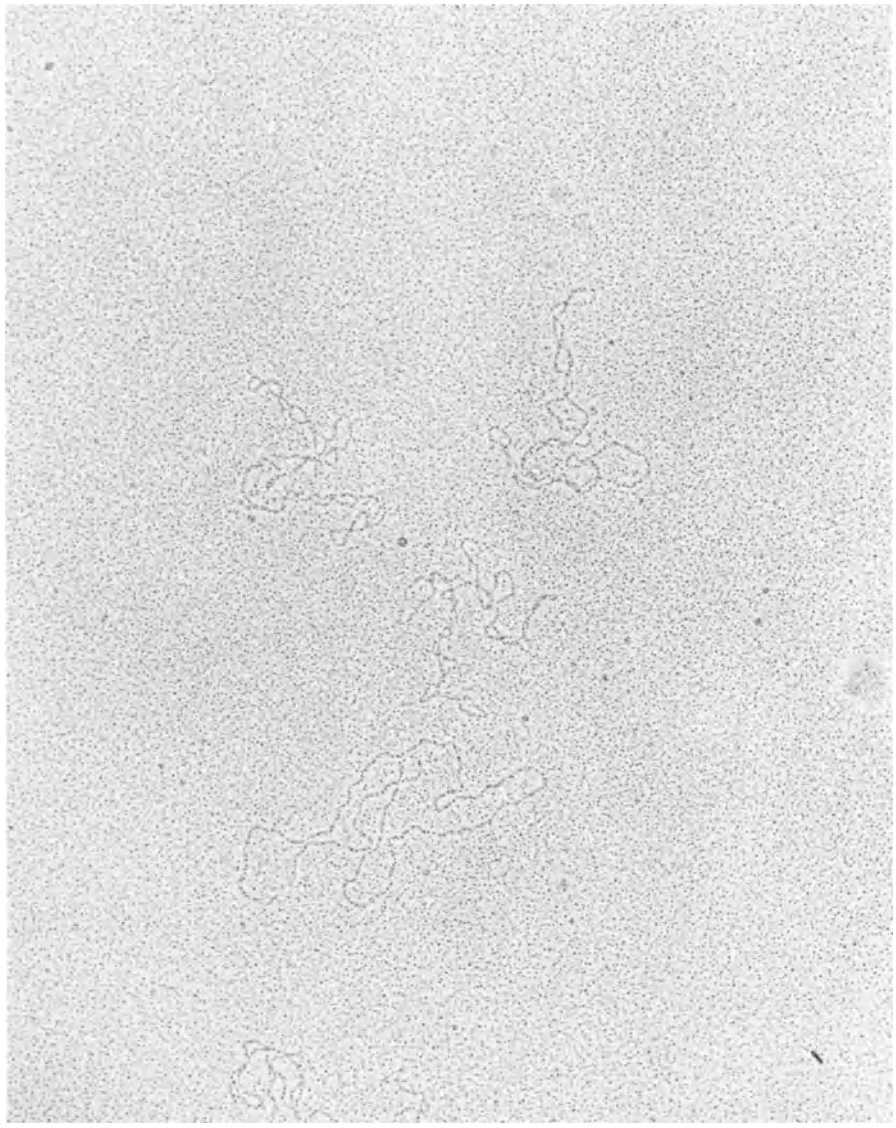


Fig. 5. Spread nucleic acid of bacteriophage ϕX174 RF DNA showing supercoils and open circles. Magnification $\times$150,000 (reduced 40% for reproduction).

ancies in the molecular weight of the specimen nucleic acid and differences in the stocks used for sequence or molecular weight analysis and stocks used for electron microscopy (such as genetic deletions and differences in spreading techniques and specimen drying in films). A comparison of the results obtained in a few studies is given in Table 3. It has generally been assumed that the average molecular weight of a

TABLE 3

Base-Pair Repeat Distances of Viral Nucleic Acids by Electron Microscopy

Specimen	Base-pair repeat distance (nm)	Reference
ϕX174 RF (DNA)	0.318 ± 0.005	Stüber and Bujard (1977)
T7 (DNA)	0.321	Stüber and Bujard (1977)
T7 restriction fragment (DNA)	0.326	Williams (1977)
λ (DNA)	0.317–0.343	Vollenweider *et al.* (1978)
R17 RF (RNA)	0.314	Granboulan and Franklin (1968)
PM2 restriction fragment (DNA)	0.326 ± 0.018	Williams (1977)
X-ray analysis of B-form paracrystals of lithium salts (DNA)	0.338	Arnott and Hukins (1973)

base-pair is 662 and that the sequence composition does not influence the spacing.

During the time that spreading methods have been used for physical mapping of viral genomes, the reports have become so numerous that they may be more interesting from the perspective of genetics than of virology or electron microscopy (see, for instance, Bukhari *et al.*, 1977). In combination with nucleic acid purification and spreading, there have been three principle locator techniques which have been used in physical mapping. These three procedures are heteroduplex formation, denaturation, and R-loop hybrid analyses. Recently, the abundant variety of restriction enzymes (Smith, 1979), which have permitted reproducible and precise cleavage of DNA at a limited number of specific nucleotide sequences, have come into increased usage as markers for linear and circular genomes.

Nomura and Benzer (1961) predicted that a more sensitive method than any other available for determining the physical size of a deletion might be direct observation in the electron microscope of unpaired loops in DNA duplex in which one strand comes from the standard type while the other comes from a deletion mutant. They suggested a method of separation and reassociation of the components of the heteroduplex in order to create an unpaired loop corresponding to the deletion. It was some time before the first reports of the visualization of deletion mutants appeared (Westmoreland *et al.*, 1969; Davis *et al.*, 1971). The general principles of physical mapping, which came into extensive use, were set down in those early ideas. Such mapping requires a two-step process in which discontinuities are first created in the nucleic acid molecule, followed by detection of these discontinuities. With an agreed-on starting or reference point, the discontinuities then

serve as signposts to locate the position and sizes of genes and spacers which characterize the nucleic acid. For the most part, detection of the discontinuties simply involves the ability to distinguish between single- and double-stranded regions in the spread nucleic acid.

Heteroduplex formation has been one of the most frequently used methods to create electron microscopically observable discontinuties that correspond to deletions and insertions that are also genetically identifiable. In the study of heteroduplex DNA of bacteriophage λ, Westmoreland *et al.* (1969) used isopycnic centrifugation to fractionate the complementary strands (l and r strands of normal and mutant DNA). Following such separation, it was possible to carry out annealing of heteroduplex on the one hand and homoduplex controls on the other. Under conditions in which incubation was carried out in a solution of 50% formamide, 0.005 M sodium bicarbonate at pH 8.6 at 4°C for at least 5 days, they found less than 2% standard deviation in the mapping measurements of deletions in the central region of the bacteriophage λ DNA molecule and of substitutions in the immunity region.

In the popular method described by Davis *et al.* (1971), these authors recognized the difficulties resulting from imperfect annealing and single-strand breaks. However, to avoid the increased handling for purification and long incubation, they devised a method which did not separate the phage ghosts or the complementary strands. They allowed only approximately 50% renaturation at 25°C, which required only 1 to 2 hr. Then, in the electron microscopic examination, they selected the easily recognized heteroduplex molecules from the aggregates and unrenatured single strands.

Partial denaturation mapping has been another widely used technique to produce unique single-stranded regions interspersed along the contour of the spread duplex molecule. Inman and Schnos (1974) based the development of two methods on the fact that A-T rich regions should melt at a lower temperature or pH than G-C rich regions. The alkaline method was found more reliable than the one utilizing thermal denaturation. Inman (1967) observed that, in DNA from bacteriophage λ, there were single-stranded regions of various but unique lengths at five to ten positions along the more contrasting double strand. A histogram was prepared from the average positions of the denatured sites as a function of map distance from a selected point. Recently, partial denaturation mapping was used to examine the correlation between the DNA regions of coliphage λ which preferentially melt at the sites specifically binding *E. coli* RNA polymerase (Vollenweider *et al.*, 1979).

Direct visualization by the BAC technique (described in Section 2.4) gave good correlation of polymerase binding in early-melting regions. This result is in agreement with indirect conclusions concerning helix stability as an important feature in the structure and function of promoter regions.

The "R-loop" technique of Thomas *et al.* (1976) is another method used for the examination of the basic organization of viral genomes by direct visualization of RNA-DNA hybrids in the electron microscope. In the formation of R-loop hybrids, double-stranded DNA was mixed with the appropriate messenger RNA and incubated under conditions of partial denaturation (formamide and temperature) that allowed some more stable RNA-DNA hybrids to form. In the regions in which the DNA was complementary to the mRNA, a loop was formed in which the thick strand was the RNA-DNA hybrid and the other was the displaced single-stranded DNA. The positions of the loops relative to some reference point in the DNA have been measured for mRNA of SV40 and Ad2 viruses. The results that have been reported relative to the processing of viral mRNAs will be discussed in Section 3.5.

3.4. Reconstitution, Assembly, and Virus Maturation

Electron microscopy has played a significant role in studies of virus reconstitution, self-assembly of viral proteins, and viral morphogenesis. In 1955, in a classic series of experiments, Fraenkel-Conrat and Williams (1955) reported that the protein and RNA components of TMV could be reconstituted in the test tube into active virus particles that were structurally indistinguishable from the original virus. The novel idea that all the information necessary for the assembly of the virus particle was inherent in its parts stimulated a number of studies, not only on the mechanism of spontaneous assembly of TMV rods, but also of other viruses of icosahedral and flexuous form as well as of the more complicated genetically regulated bacteriophage morphogenesis. In a previous volume of this series, Richards and Williams (1976) reviewed the experiments up to that time on the modes of aggregation of the subunits of TMV under a variety of conditions in both the presence and absence of the nucleic acid. Lauffer and Stevens (1968) had demonstrated that this polymerization was an endothermic process, and Caspar (1963) suggested that the polymerization of the TMV protein subunits into a helix could be a representative example of the self-assembly of virus particles in general. Caspar (1963) also considered the

"initiation" of the TMV rods as a process different and separate from "elongation." Self-assembly of TMV protein is shown in Fig. 6.

Recent studies by Lebeurier *et al.* (1977) and Butler *et al.* (1977) used separate techniques to examine by electron microscopy the tails of unencapsidated RNA during reconstitution. The results of both experiments showed that the assembly proceeds rapidly from an internal

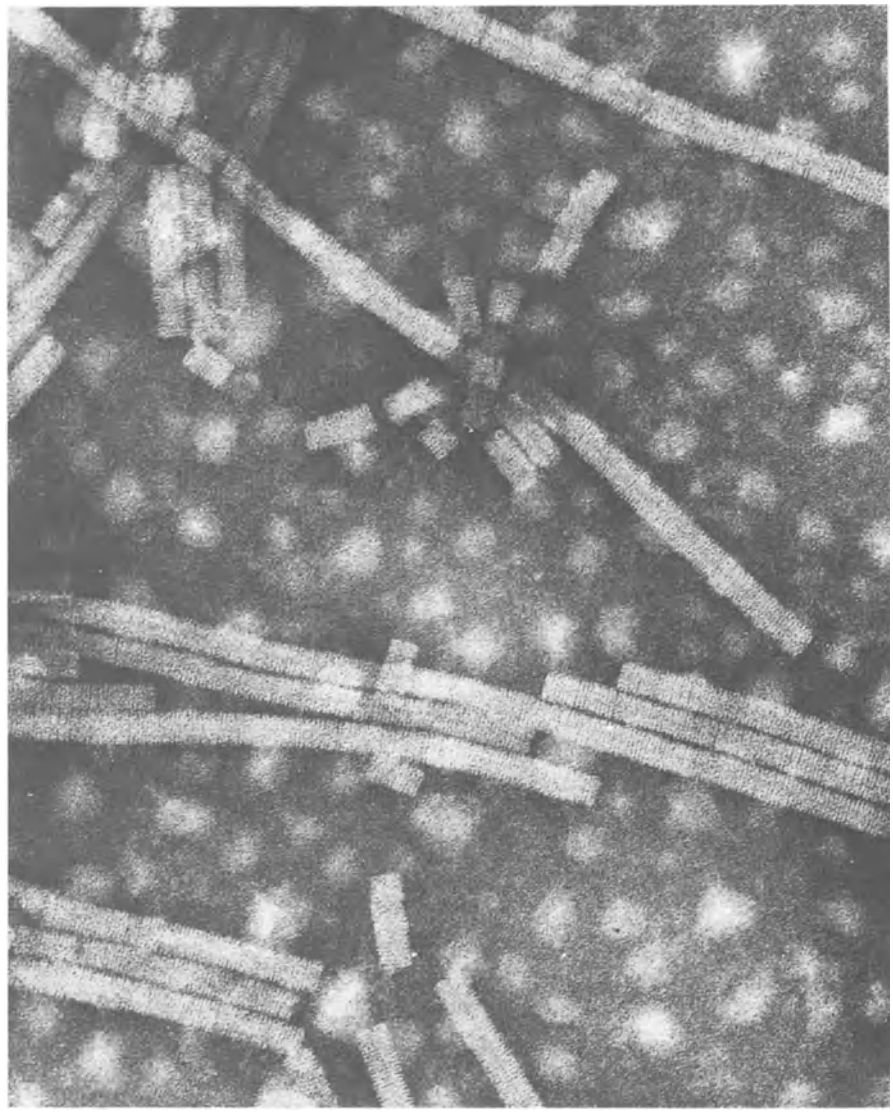


Fig. 6. Negative stain preparation of tobacco mosaic virus protein, polymerized. Magnification ×300,000 (reduced 39% for reproduction).

initiation site to the 5′-OH end of the viral RNA, followed by a slower encapsidation from the initiation site to the 3′-OH end.

The self-assembly of the flexuous papaya mosaic virus (PMV), which is a member of the potato virus X family, has been studied by Bancroft and co-workers. Erickson and Bancroft (1978) found that PMV could be reconstituted in two distinct steps, a rapid initiation phase and a slower elongation phase, which could be examined separately by electron microscopy by controlling the incubation temperature. Particles about 50 nm long were formed in the initiation step at either 1°C or 25°C, whereas the elongation phase did not proceed at 1°C but did at 25°C. Abouhaidar and Bancroft (1978) found that the assembly process of PMV started, unlike that of TMV, near the 5′-OH end of the RNA.

The *in vitro* self-assembly of spherical plant viruses and bacteriophage has also been studied by electron microscopy, primarily with the aid of the negative staining technique. For example, self-assembly of icosahedral viruses such as brome mosaic virus (BMV) and cowpea chlorotic mosaic virus (CCMV) has been attempted. A variety of bizarre physical forms including tubes, small spheres, ellipsoids, and double-walled spheres have been obtained with the capsomers in the reaggregated assemblies. The interchanging of the protein and the RNA from several different viruses in the reconstitution has always yielded progeny virus genetically the same as the RNA-donating parent, and the electrophoretic mobility and antigenic properties have been identical to the protein-donating parent. Bancroft (1970) has reviewed the self-assembly of spherical plant viruses, and Hohn and Hohn (1970) have reviewed that of the simple RNA bacteriophages.

Closely related to the reconstitution experiments have been a very large number of electron microscopic studies of the steps of *in vivo* virus morphogenesis, particularly of tailed bacteriophage. One of the questions specifically addressed as long ago as 1953 (Levinthal and Fisher, 1953) has been the problem of how the DNA could be packed into a preformed, empty phage head. Thomas (1974) and Chattoraj and Inman (1974) investigated with phages λ, P2, 186, and P4 whether there was specificity in the attachment of a particular end of the DNA to the phage tail. Other recent studies with phage λ have been by Katsura and Tsugita (1977) and Zachary and Simon (1977); for the T-even bacteriophages, by Carrascosa and Kellenberger (1978), Carrascosa (1978), and Hsiao and Black (1978); for bacteriophage T7, by Roeder and Sadowski (1977) and Masker *et al.* (1978); for phage P22, by Poteete and King (1977); and for φX174, by Fujisawa and Hayashi (1977). A recent review of virus assembly was presented by Casjens and King

(1975). The problem of DNA folding within phage heads has been examined by Laemmli (1975), Richards *et al.* (1973), and Chattoraj *et al.* (1978).

3.5. Growth and Replication of Viruses

Many of the cytological and quantitative techniques described in Sections 2.2 (ultrathin sectioning), 2.5 (freeze etching), and 2.6 (particle counting) have been used to examine different elements of viral growth such as attachment, penetration, uncoating, and release of mature virus. Some of the studies of bacteriophage attachment and growth preceded by quite a few years the comparable examinations of animal, plant, and insect viruses. Similarly, many of the ideas of simple plant virus and bacteriophage assembly discussed in Section 3.4 established principles which eventually were applied to more intricate animal viruses. The complexity and compartmentalization of the host cells of the animal and plant viruses has necessitated more elaborate experimentation, such as immunocytochemical localization of virus-related structures.

Historically, the early and the late events of the viral one-step growth curve have been considered separately from the middle period simply because it was an "eclipse" period. The virus could not be demonstrated by bioassay during this period, nor could virus components be identified by electron microscopy (Levinthal and Fisher, 1952) even though extensive viral biosynthesis was obviously taking place. For this discussion, it might be helpful to use this rather loose division of the sequence of events which characterize virus growth because the techniques which have been most useful for the early and late events have been different from those most useful for studies of the middle (eclipse) period.

Some of the cytological techniques used in the electron microscopic studies of the early events of virus infection have been used to examine the interaction of virus particles with cell membranes, the cell compartments for virus localization, and the identification of the site of synthesis of virus-specific antigens. For instance, such studies are exemplified by the attempts to distinguish fusion of virus particles to cell membranes from pinocytosis for poliovirus (picornavirus) (Dales *et al.*, 1965; Dunnebacke *et al.*, 1969; Levinthal *et al.*, 1969; Mandel, 1962). Membrane fusion has been examined as a mechanism for SV40 entry into different cellular compartments (Maul *et al.*, 1978). Other examples of studies of early events have been the attempts at the cellular

localization of the uncoating of the virus particles for an insect virus (Granados, 1978) and for a picornavirus (Eggers *et al.*, 1979).

During the late phase of virus growth, many of the studies have examined the maturation, envelopment, and budding of virus particles. As an example, the study by Steere and Erbe (1979) illustrated in Fig. 3 shows particles of potato yellow dwarf virus in the space between the two nuclear membranes. These particles bud out of the nucleoplasm through the inner nuclear membrane and acquire an outer shell as they do so. Also, spikes develop on the outer surface of this membrane (apparently only where it surrounds a virion). A similar study on the localization of wheat striate mosaic virus in its leafhopper vector has recently been carried out by Bell *et al.* (1978). Biogenesis of flu (Lohmeyer *et al.*, 1979; Caliguiri and Holmes, 1979) and vaccinia virus (Dales *et al.*, 1978) are other recent examples of this kind of electron microscopic characterization. Figure 7 is an electron micrograph showing mouse mammary tumor cells with extracellular and budding virus particles.

The spreading methods described in Section 2.4 have been most useful for studies of the eclipse period of virus replication. During this period, the changes in the viral nucleic acids which can be observed by the spreading techniques are noteworthy. These are principally (1) replication and recombination, (2) transcription and translation, and (3) processing and splicing. The several advantages of carrying out such studies with viral nucleic acids, based on their unique small size, and coupled with the techniques of rapid sequence analysis and specific cleavage with restriction enzymes, has recently made this one of the most active current areas of investigation in molecular biology.

There have been several mysteries in the replication of viral nucleic acids and in the closely related process of recombination. For instance, there is the question of how copies of the genetic material in the form of single-stranded circles can be made by a semiconservative mechanism. Another question has been the direction of replication, or whether replication is bidirectional. The life cycle of bacteriophage ϕX174 has been the subject of a number of electron microscope studies. A recent study of the replication events of phage ϕX174 DNA was preformed by Koths and Dressler (1978). Their procedure allowed a quantitative examination of the intracellular forms of viral DNA free from contamination by host DNA with a one-step density-labeling protocol. The results showed an accumulation of duplex rings of two types early in the life cycle: a rolling circle with a single-stranded tail, and a single-stranded circle that was partially duplex. Late in the life cycle, only the rolling circle was found as the virus particles were assembled. It was

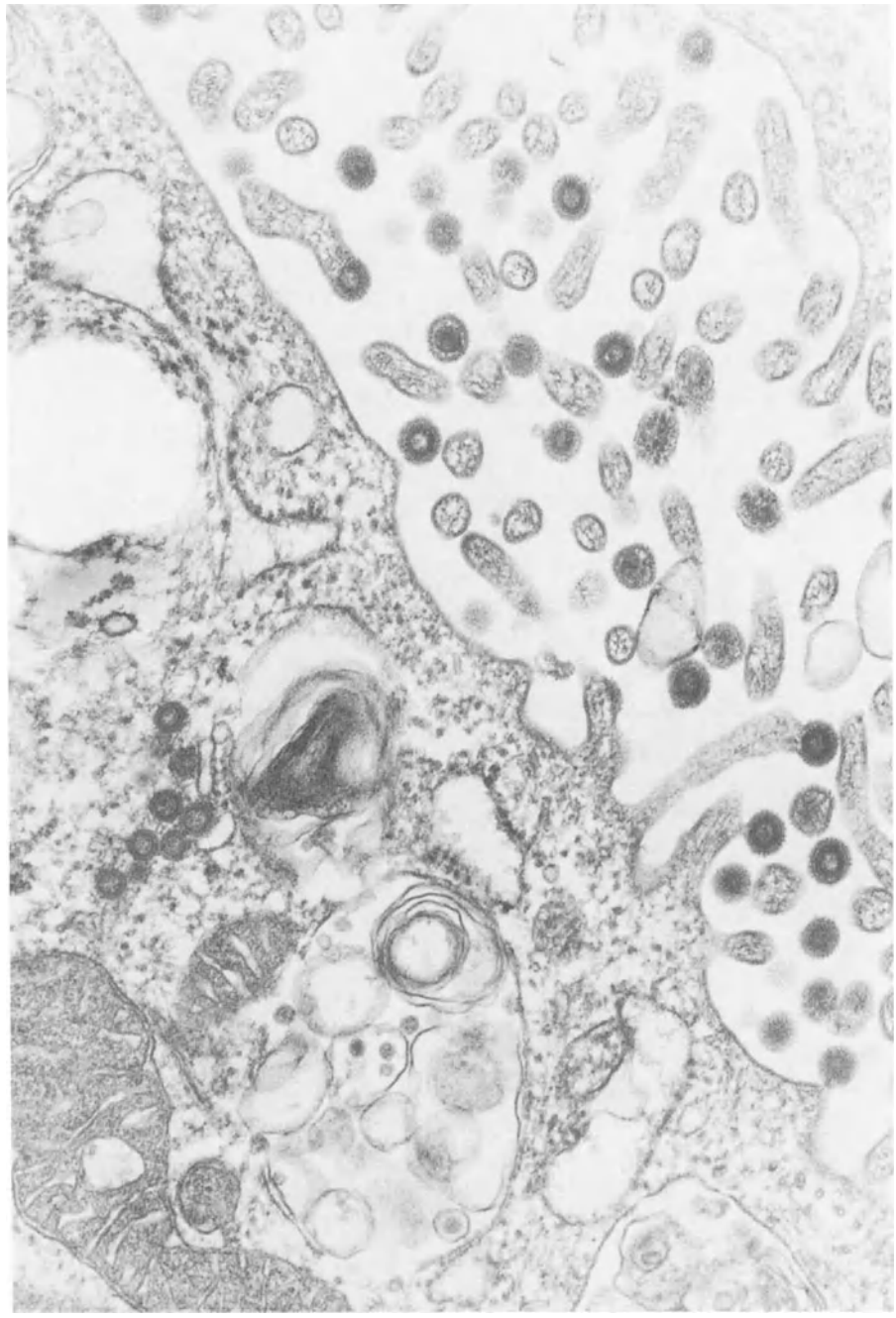


Fig. 7. Thin section of mouse mammary tumor cell line showing extracellular and budding B particles. Magnification ×30,000 (reduced 24% for reproduction). Courtesy of R. Shiurba, B. Taggart, and D. Pitelka.

concluded that the rolling circle was the replicating intermediate. Studies have also been done on phages S13 and G4 (Thompson *et al.*, 1976) and on the RNA bacteriophage R17 (Thach and Thach, 1973).

Recombination has been studied using the spreading for electron microscopy of recombination intermediates of bacteriophage lambda. Valenzuela and Inman (1975) photographed the DNA molecules after partial denaturation and showed them to be physically connected at homologous positions. Several other studies with virus-infected cells have examined the evidence related to the model of Holliday (1974) for DNA recombination. These include the studies of the "figure 8" structures from ϕX174-infected cells described by Thompson *et al.* (1975) and others cited in the review by Fisher and Williams (1979).

For a number of reasons, research with the electron microscope has been extremely active in studies of RNA synthesis, particularly with *E. coli* bacteriophages. For one thing, the RNA polymerase of the host cells is a large enzyme and has been well characterized. In addition, binding of the polymerase to the DNA and the structure and function of promoters has been analyzed, particularly for bacteriophages lambda and T7. Furthermore, the complete base sequences of the nucleic acids of the small bacteriophages such as ϕX174, S13, G4, and fd have been published. Electron microscope studies of binding complexes between RNA polymerase and T7 DNA were carried out by Williams and Chamberlin (1977) and between the polymerase and ϕX174 DNA replicative form by Rassart *et al.* (1979) and Williams and Fisher (1979) (see Fig. 8). Methods for visualizing *in vitro* synthesized RNA still attached to the DNA template have been described for T7 and SV40 by Delius *et al.* (1973) and for ϕX174 supercoils by Williams and Fisher (1979). Recent discussions which also dealt with electron microscopy of transcription complexes can be found in Fisher and Williams (1979) and Spencer (1979).

The mechanisms for regulation of gene expression studied through use of electron microscopy of viral specimens has been a subject of active research. However, there seems to be a distinct difference between the strategy of bacteriophage and animal virus genome expression. In the latter case, extremely accurate splicing of mRNA has been reported, whereas mechanisms for processing genomes have not been described for procaryotic systems. Nevertheless, there has been some electron microscopy of secondary structure in phage nucleic acids. A study by Jacobson (1976) examined the hairpin loop formation in MS2 phage RNA with increasing Mg^{2+} concentration and compared the results of the loop locations with the sequence data. Edlind and Bassel (1977) also examined the existence of loops for the RNA of

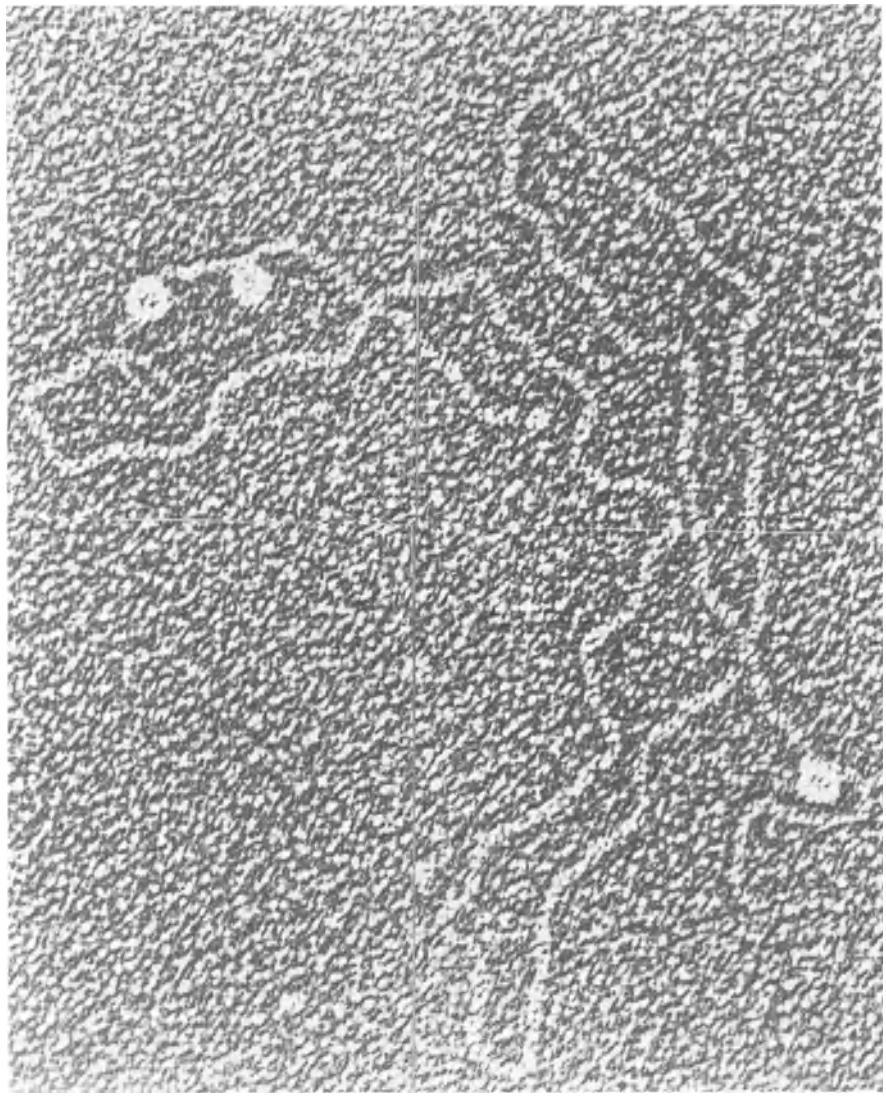


Fig. 8. Binary complexes between *E. coli* RNA polymerase and bacteriophage ϕX174 RF DNA. Magnification $\times$400,000 (reduced 30% for reproduction).

phages f2, Qβ, and PP7. The electron microscopic characterization of the spliced RNAs of adenovirus 2 and SV40 has been very effective in confirming the widely held behief that the mRNAs of viruses (which infect eucaryotic cells) are generated by the processing of larger, primary transcripts. The R-loop method of preparing hybrids of double-stranded DNA and the mRNA of interest has been a very useful

tool in these studies (Chow and Broker, 1979; Chow *et al.*, 1977; Flint, 1977; Acheson, 1976; Berget *et al.*, 1978).

4. CONCLUSION

In attempting to review the actual and potential contributions of electron microscopy to virology, one is handicapped by the available space to describe the magnitude of the accomplishments and the technical ingenuity of the workers. One might say that, for the first 40 years, the electron microscope must have been made for virology. Considerable progress has been made in specimen preparation techniques that make possible resolution of details not far from the resolution limit of currently available instruments and which allow routine characterization of viruses and viral components. This is not to say that interesting challenges in perfecting techniques do not remain. For instance, little is understood of the mechanism of the glow discharge used for making specimen films hydrophilic. Reproducible and uniform spreading of viruses and enzymes in negative stain is far from faultless. The avoidance of alterations by surface tension forces, by nonaqueous solvents, and by fixatives and stains still needs attention. Procedures to achieve orderly alignment of viral particles such as the natural order found in the arrangement of the subunits of the purple membrane (Blaurock and Stoeckenius, 1971) could increase resolution through the superposition of repetitive images. Specific electron stains for the study of viral components at high resolution have yet to be developed.

The recently developed methods for rapid sequencing of nucleic acids and the use of the highly specific restriction endonucleases most likely will play a significant role in the course of future studies of viral nucleic acids and their genetic analysis, replication, transcription, translation, and regulation. It will be interesting to see how the direct visualization in the electron microscope of these structures will influence our understanding of functions and mechanisms.

5. REFERENCES

Abercrombie, M., Heaysman, J. E. M., and Pegrum, S. M., 1971, The locomotion of fibroblasts in culture. IV. Electron microscopy of the leading lamella, *Exp. Cell Res.* **67**:359.

Abermann, R., Salpeter, M. M., and Bachmann, L., 1972, High resolution shadowing, in: *Principles and Techniques of Electron Microscopy. Biological Applications*, Vol. 2 (M. A. Hayat, ed.), Van Nostrand Reinhold, New York.

Abouhaidar, M., and Bancroft, J. B., 1978, The initiation of papaya mosaic virus assembly, *Virology* **90**:54.

Acheson, N. H., 1976, Transcription during productive infection with polyoma virus and simian virus 40, *Cell* **8**:1.

Anderson, T. F., 1951, Techniques for the preservation of three-dimensional structure in preparing specimens for the electron microscope, *Tr. N.Y. Acad. Sci.* **13**:130.

Arnberg, A. C., and Arwert, F., 1976, DNA-protein complex in circular DNA from *Bacillus* bacteriophage GA-1, *J. Virol.* **18**:783.

Arnott, S., and Hukins, D. W. L., 1973, Refinement of the structure of B-DNA and implications for the analysis of X-ray diffraction data from fibers of biopolymers, *J. Mol. Biol.* **81**:93.

Backus, R. C., and Williams, R. C., 1949, Small spherical particles of exceptionally uniform size, *J. Appl. Phys.* **20**:98.

Backus, R. C., and Williams, R. C., 1950, The use of spraying methods and of volatile suspending media in the preparation of specimens for electron microscopy, *J. Appl. Phys.* **21**:11.

Bancroft, J. B., 1970, The self-assembly of spherical plant viruses, *Adv. Virus Res.* **16**:99.

Bell, C. D., Omar, S. A., and Lee, P. E., 1978, Electron microscopic localization of wheat striate mosaic virus in its leafhopper vector *Endria inimica*, *Virology* **86**:1.

Berget, S. M., Berk, A. J., Harrison, T. H., and Sharp, P. A., 1977, Spliced segments at the 5′ termini of adenovirus-2 late mRNA: A role for heterogeneous nuclear RNA in mammalian cells, *Cold Spring Harbor Symp. Quant. Biol.* **42**:523.

Bernhard, W., 1964, Fine structural lesions induced by viruses, Ciba Foundation Symposium on Cellular Injury.

Bils, R. F., and Hall, C. E., 1962, Electron microscopy of wound tumor virus, *Virology* **17**:123.

Blaurock, A. E., and Stoeckenius, W., 1971, Structure of the purple membrane, *Nature (London) New Biol.* **233**:152.

Bradley, D. E., 1954, Evaporated carbon films for use in electron microscopy, *Br. J. Appl. Phys.* **5**:65.

Branton, D., Bullivant, S., Gilula, N. B., Karnovsky, M. J., Moore, H., Muhlethaler, K., Northcote, D. H., Packer, L., Satir, B., Satir, P., Speth, V., Staehlin, L. A., Steere, R. L., and Weinstein, R. S., 1975, Freeze-etching nomenclature, *Science* **190**:54.

Brenner, S., and Horne, R. W., 1959, A negative staining method for high resolution electron microscopy of viruses, *Biochim. Biophys. Acta* **34**:103.

Brinkley, B. R., Murphy, P., and Richardson, L. C., 1967, Procedure for embedding *in situ* selected cells cultured *in vitro*, *J. Cell Biol.* **35**:279.

Bukhari, A. I., Shapiro, J. A., and Adhya, S. L., eds., 1977, *DNA Insertion Elements, Plasmids, and Episomes*, 782 pp., Cold Spring Harbor, N.Y.

Burton, E. F., and Kohl, W. H., 1946, *The Electron Microscope. An Introduction to its Fundamental Principles and Applications*, 325 pp., Reinhold, New York.

Butler, P. J. G., Finch, J. T., and Zimmern, D., 1977, Configuration of tobacco mosaic virus RNA during virus assembly, *Nature (London)* **265**:217.

Cairns, J., 1960, The initiation of vaccinia infection, *Virology* **11**:603.

Caliguiri, L. A., and Holmes, K. V., 1979, Host-dependent restriction of influenza virus maturation, *Virology* **92**:15.

Caro, L. G., 1962, High resolution autoradiography. II. The problem of resolution, *J. Cell Biol.* **15**:189.

Caro, L. G., and Van Tubergen, R. P., 1962, High resolution autoradiography. I. Methods, *J. Cell Biol.* **15**:173.

Carrascosa, J. L., 1978, Head maturation pathway of bacteriophages T4 and T2. IV. *In vitro* transformation of T4 head-related particles produced by mutants in gene 17 to capsid-like structures, *J. Virol.* **26**:420.

Carrascosa, J. L., and Kellenberger, E., 1978, Head maturation pathway of bacteriophages T4 and T2. III. Isolation and characterization of particles produced by mutants in gene 17, *J. Virol.* **25**:831.

Casjens, S., and King, J., 1975, Virus assembly, *Annu. Rev. Biochem.* **44**:555.

Caspar, D. L. D., 1963, Assembly and stability of the tobacco mosaic virus particle, *Adv. Protein Chem.* **18**:37.

Caspar, D. L. D., and Klug, A., 1962, Physical principles in the construction of regular viruses, *Cold Spring Harbor Symp. Quant. Biol.* **27**:1.

Chattoraj, D. K., and Inman, R. B., 1974, Location of DNA ends in P2, 186, P4 and lambda bacteriophage heads, *J. Mol. Biol.* **87**:11.

Chattoraj, D. K., Gosule, L. C., and Schellman, J. A., 1978, DNA condensation with polyamines. II. Electron microscopic studies, *J. Mol. Biol.* **121**:327.

Chow, L. T., and Broker, T., 1979, The spliced RNAs of adenovirus: Electron microscopic characterization, in: *Proc. 37th Ann. Mt. E.M.S.A.* (G. W. Bailey, ed.), pp. 62–65, Claitor's Publishing Division, Baton Rouge.

Chow, L. T., Roberts, J. M., Lewis, J. B., and Broker, T. R., 1977, A map of cytoplasmic RNA transcripts from lytic adenovirus type 2 determined by electron microscopy of RNA:DNA hybrids, *Cell* **11**:819.

Cohen, A. L., 1974, Critical point drying, in: *Principles and Techniques of Scanning Electron Microscopy: Biological Applications*, Vol. 1 (M. A. Hayat, ed.), Van Nostrand Reinhold, New York.

Crane, H. R., 1944, Direct centrifugation onto electron microscope specimen films, *Rev. Sci. Instrum.* **15**:253.

Crick, F. H. C., and Watson, J. D., 1956, Structure of small viruses, *Nature* (*London*) **177**:473.

Croissant, O., Dauguet, C., Jeanteur, P., and Orth, G., 1972, Application de la technique d'hybridization moleculaire *in situ* a la mise en evidence au microscope electronique de la replication vegetative de l'ADN viral dans les papillomes provoques par le virus de Shope chez le lapin cottontail, *C. R. Acad. Sci.* **274**:614.

Dalen, H., and Nevalainen, T. J., 1968, Direct epoxy embedding for vertical sectioning of cells grown as a monolayer on millipore filter, *Stain Tech.* **43**:217.

Dales, S., 1963, The uptake and development of vaccinia virus in strain L cells followed with labeled viral deoxyribonucleic acid, *J. Cell Biol.* **18**:51.

Dales, S., Eggers, H. J., Tamm, I., and Palade, G. E., 1965, Electron microscopic study of the formation of poliovirus, *Virology* **26**:379.

Dales, S., Milovanovitch, V., Poggo, B. G. T., Weintraub, S. B., Huima, T., Wilton, S., and McFadden, G., 1978, Biogenesis of vaccinia: Isolation of conditional lethal mutants and electron microscopic characterization of their phenotypically expressed defects, *Virology* **84**:403.

Davis, R. W., Simon, M., and Davidson, N., 1971, Electron microscopic heteroduplex methods for mapping regions of base sequence homology in nucleic acids, in:

Methods in Enzymology, Vol. 21 (L. Grossman, and K. Moldave, eds.), p. 413, Academic Press, New York.

Dawes, C. J., 1971, *Biological techniques in electron microscopy*, Barnes and Noble, New York.

de Harven, E., Beju, D., Evenson, D. P., Basu, S., and Schidlousky, G., 1973, Structure of critical point dried oncornaviruses, *Virology* **55**:535.

Delbrück, M., 1946, Bacterial viruses or bacteriophages, *Biol. Rev.* **21**:30.

Delius, H., Westphal, H., and Axelrod, N., 1973, Length measurements of RNA synthesized *in vitro* by *Escherichia coli* RNA polymerase, *J. Mol. Biol.* **74**:677.

Douglas, W. H. J., and Elser, J. E., 1972, A method for *in situ* embedding of cultured cells grown on plastic surfaces, *In Vitro* **8**:26.

Dubochet, J., Ducommun, M., Zollinger, M., and Kellenberger, E., 1971, A new preparation method for dark-field electron microscopy of biomacromolecules, *J. Ultrastruct. Res.* **35**:147.

Dunnebacke, T. H., and Kleinschmidt, A. K., 1967, Ribonucleic acid from reovirus as seen in protein monolayers by electron microscopy, *Z. Naturforsch.* **22b**:159.

Dunnebacke, T. H., Levinthal, J. D., and Williams, R. C., 1969, Entry and release of poliovirus as observed by electron microscopy of cultured cells, *J. Virol.* **4**:505.

Edlind, T. D., and Bassel, A. R., 1977, Secondary structure of RNA from bacteriophage f2, Qβ, and PP7, *J. Virol.* **24**:135.

Eggers, H. J., Bode, B., and Brown, D. T., 1979, Cytoplasmic localization of the uncoating of picornaviruses, *Virology* **92**:211.

Erickson, J. W., and Bancroft, J. B., 1978, The self-assembly of papaya mosaic virus, *Virology* **90**:36.

Evenson, D. P., 1977, Electron microscopy of viral nucleic acids, in: *Methods in Virology*, Vol. 6 (K. Maramorosch and H. Koprowski, eds.), p. 219, Academic Press, New York.

Fenner, F., 1968, The biology of animal viruses, *Molecular and Cellular Biology*, Vol. I, Academic Press, New York.

Fernandez-Moran, H., 1953, A diamond knife for ultrathin sectioning, *Exp. Cell Res.* **5**:255.

Finch, J. T., and Crawford, L. V., 1975, Structure of small DNA-containing animal viruses, in: *Comprehensive Virology*, Vol. 5 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 119, Plenum Press, New York.

Fisher, H. W., and Cooper, T. W., 1967, Electron microscope studies of the microvilli of Hela cells, *J. Cell Biol.* **34**:569.

Fisher, H. W., and Williams, R. C., 1979, Electron microscopic visualization of nucleic acids and of their complexes with proteins, *Annu. Rev. Biochem.* **48**:649.

Flint, S. J., 1977, The topography and transcription of the adenovirus genome, *Cell* **10**:153.

Fraenkel-Conrat, H., and Williams, R. C., 1955, Reconstitution of active tobacco mosaic virus from its inactive protein and nucleic acid components, *Proc. Natl. Acad. Sci. USA* **41**:690.

Fraenkel-Conrat, H., 1974, *Comprehensive Virology*, Vol 1, *Descriptive Catalogue of Viruses* (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 103, Plenum Press, New York.

Friedman, M. H., 1970, A reevaluation of the Markham rotation technique using model systems, *J. Ultrastruct. Res.* **32**:226.

Fujisawa, H., and Hayashi, M., 1977, Assembly of bacteriophage ϕX174: Identification

of a virion capsid precursor and proposal of a model for the functions of bacteriophage gene products during morphogenesis, *J. Virol.* **24**:303.

Geuskens, M., 1974, ^{3}H-Actinomycin D binding to DNA localization in ultrathin sections of plastic-embedded biological materials, *J. Ultrastruct. Res.* **47**:179.

Geuskens, M., and May, E., 1974, Ultrastructural localization of SV40 viral DNA in cells, during lytic infection, by *in situ* molecular hybridization, *Exp. Cell Res.* **87**:175.

Glauert, A., 1972, *Practical Methods in Electron Microscopy*, American Elsevier, New York.

Glauert, A. M., Rogers, G. E., and Glauert, R. H., 1956, A new embedding medium for electron microscopy, *Nature (London)* **178**:803.

Gonda, M. A., Gilden, R. V., Oroszlan, S., Hager, H., and Hsu, K. C., 1978, Immunolatex spheres for cell and virion surface labeling in the electron microscope, *Virology* **86**:572.

Granados, R. R., 1978, Early events in the infection of *Heliothis zea* midget cells by a baculovirus, *Virology* **90**:170.

Granboulan, N., and Franklin, R. M., 1968, Replication of bacteriophage ribonucleic acid: Analysis of the ultrastructure of the replicative form and the replicative intermediate of bacteriophage R17, *J. Virol.* **2**:129.

Griffith, J. D., 1973, Electron microscopic visualization of DNA in association with cellular components, *Methods Cell Biol.* **7**:129.

Hall, C. E., 1955, Electron densitometry of stained virus particles, *J. Biophys. Biochem. Cytol.* **1**:1

Hall, C. E., 1966, *Introduction to Electron Microscopy*, McGraw-Hill, New York.

Hart, R. G., 1963, A method for shadowing electron microscopic specimens with tungsten, *J. Appl. Phys.* **34**:434.

Haschemeyer, R. H., and Myers, R. J., 1972, Negative staining, in: *Principles and Techniques of Electron Microscopy*, Vol. 2 (M. A. Hayat, ed.), p. 101, Van Nostrand Reinhold, New York.

Hatta, T., and Francki, R. I. B., 1979, The fine structure of chloris striate mosaic virus, *Virology* **92**:428.

Hayat, M. A., 1970, *Principles and Techniques of Electron Microscopy: Biological Applications*, Vol. 1, Van Nostrand Reinhold, New York.

Hayat, M. A., 1972, *Basic Electron Microscopy Techniques*, Van Nostrand Reinhold, New York.

Heide, H. G., 1965, Techniques for the prevention of contamination of the object, *Lab. Invest.* **14**:1140.

Heinmets, F., 1949, Modification of silica replica techniques for study of biological membranes and application of rotary condensation in electron microscopy, *J. Appl. Phys.* **20**:384.

Henderson, W. J., and Griffiths, K., 1972, Shadow casting and replication, in: *Principles and Techniques of Electron Microscopy*, Vol. 2 (M. A. Hayat, ed.), p. 151, Van Nostrand Reinhold, New York.

Highton, P. J., and Whitfield, M., 1974, The control of the configuration of nucleic acid molecules deposited for electron microscopy, by ionic bombardment of carbon films, *J. Microsc.* **100**:299.

Hirsh, J., and Schleif, R., 1976, High resolution electron microscopic studies of genetic regulation, *J. Mol. Biol.* **108**:471.

Hohn, T., and Hohn, B., 1970, Structure and assembly of simple RNA bacteriophages, *Adv. Virus Res.* **16**:43.

Holliday, R., 1974, Molecular aspects of genetic exchange and gene conversion, *Genetics* **78**:273.

Holmes, K. C., Stubbs, G. J., Mandelkow, E., and Gallwitz, U., 1975, Structure of tobacco mosaic virus at 6.7 Å resolution, *Nature (London)* **254**:192.

Holt, S. J., and Hicks, R. M., 1962, in: *The Interpretation of Ultrastructure* (R. J. C. Harris, ed.), p. 193, Academic Press, New York.

Horne, R. W., and Ronchetti, I. P., 1974, A negative staining–carbon film technique for studying viruses in the electron microscope. I. Preparative procedures for examining icosahedral and filamentous viruses, *J. Ultrastruct. Res.* **47**:361.

Horne, R. W., and Wildy, P., 1961, Symmetry in virus architecture, *Virology* **15**:348.

Horne, R. W., Ronchetti, I. P., and Hobart, J. M., 1975, A negative staining–carbon film technique for studying viruses in the electron microscope. II. Application to adenovirus type 5, *J. Ultrastruct. Res.* **51**:233.

Hsiao, C. L., and Black, L. W., 1978, Head morphogenesis of bacteriophage T4. III. The role of gene 20 in DNA packaging, *Virology* **91**:26.

Huxley, H. E., 1958, Some aspects of staining of tissue for sectioning, *J. R. Microsc. Soc.* **78**:30.

Inman, R., 1967, Denaturation maps of the left and right sides of the λ DNA molecule determined by electron microscopy, *J. Mol. Biol.* **28**:103.

Inman, R., and Schnos, M., 1974, Denaturation mapping of DNA, in: *Principles and Techniques of Electron Microscopy*, Vol. 4 (M. A. Hayat, ed.), p. 64, Van Nostrand Reinhold, New York.

Isaacs, A., 1957, Particle counts and infectivity titrations for animal viruses, *Adv. Virus Res.* **4**:111.

Isaacson, M. S., 1977, Specimen damage in the electron microscope, in: *Principles and Techniques of Electron Microscopy*, Vol. 7, p. 1, Van Nostrand Reinhold, New York.

Jacobson, A. B., 1976, Studies on secondary structure of single-stranded RNA from bacteriophage MS2 by electron microscopy, *Proc. Natl. Acad. Sci. USA* **73**:307.

Karnovsky, M. J., 1965, A formaldehyde-glutaraldehyde fixative of high osmolality for use in electron microscopy, *J. Cell. Biol.* **27**:137A.

Katsura, I., and Tsugita, A., 1977, Purification and characterization of the major protein and the terminator protein of the bacteriophage λ tail, *Virology* **76**:129.

Kausche, G. A., Pfankuch, E., and Ruska, H., 1939, Examination of plant viruses with the high power electron microscope, *Naturwissenschaften* **27**:292.

Kay, D. (ed.), 1965, *Techniques for Electron Microscopy*, Blackwell, Oxford.

Kellenberger, E., and Arber, W., 1957, Electron microscopical studies of phage multiplication. I. A method for quantitative analysis of particle suspensions, *Virology* **3**:245.

Kirschner, M. W., Honig, L. S., and Williams, R. C., 1975, Quantitative electron microscopy of microtubule assembly *in vitro*, J. Mol. Biol. **99**:263.

Kiselev, N. A., and Klug, A., 1969, The structure of viruses of the papilloma-polyoma type. V. Tubular variants built of pentamers, *J. Mol. Biol.* **40**:155.

Kleinschmidt, A. K., 1968, Monolayer techniques in electron microscopy of nucleic acid molecules, in: *Methods in Enzymology*, Vol. 12 (L. Grossman and K. Moldave, eds.), Part B, p. 361, Academic Press, New York.

Kleinschmidt, A. K., and Zahn, R. K., 1959, Uber Deoxyribonucleinsäure—Molekeln in Protein—Mischfilmen, *Z. Naturforsch.* **14b**:770.

Kleinschmidt, A. K., Burton, A., and Sinsheimer, R. L., 1963, Electron microscopy of the replicative form of the DNA of the bacteriophage φX174, *Science* **142**:961.

Klug, A., and Finch, J. T., 1968, Structure of viruses of the papilloma-polyoma type. IV. Analysis of tilting experiments in the electron microscope, *J. Mol. Biol.* **31**:1.

Koehler, J. K., 1972, The freeze-etching technique, in: *Principles and Techniques of Electron Microscopy*, Vol. 2 (M. A. Hayat, ed.), p. 53, Van Nostrand Reinhold, New York.

Koller, T., Sogo, J. M., and Bujard, H., 1974, An electron microscopic method for studying nucleic acid protein complexes. Visualization of RNA polymerase bound to the DNA of bacteriophages T7 and T3, *Biopolymers* **13**:995.

Koths, K., and Dressler, D., 1978, Analysis of the ϕXDNA replication cycle by electron microscopy, *Proc. Natl. Acad. Sci. USA* **75**:605.

Laemmli, U. K., 1975, Characterization of DNA condensates induced by poly(ethylene oxide) and polylysine, *Proc. Natl. Acad. Sci. USA* **72**:4288.

Lang, D., 1972, Nucleic acid molecules prepared by monolayer techniques, in: *Proc. 30th Ann. Mtg. E.M.S.A.* (C. J. Arceneaux, ed.), pp. 178–179, Claitor's Publishing Division, Baton Rouge.

Lang, D., Kleinschmidt, A. K., and Zahn, R. K., 1964, Konfiguration und längenverteilung von DNA-molekülen in lösung, *Biochim. Biophys. Acta* **88**:142.

Latta, H., and Hartman, J. F., 1950, Use of a glass edge in thin sectioning for electron microscopy, *Proc. Soc. Exp. Biol. Med.* **74**:436.

Lauffer, M. A., and Stevens, C. L., 1968, Structure of the tobacco mosaic virus particle; polymerization of tobacco mosaic virus protein, *Adv. Virus Res.* **13**:1.

Lebeurier, G., Nicolaieff, A., and Richards, K. E., 1977, Inside-out model for self-assembly of tobacco mosaic virus, *Proc. Natl. Acad. Sci. USA* **74**:149.

Levinthal, C., and Fisher, H. W., 1952, The structural development of a bacterial virus, *Biochim. Biophys. Acta* **9**:419.

Levinthal, C., and Fisher, H. W., 1953, Maturation of phage and the evidence of phage precursors, *Cold Spring Harbor Symp. Quant. Biol.* **18**:29.

Levinthal, J. D., Cerottini, J. C., Ahmad-Zadeh, C., and Wicker, R., 1967, The detection of intracellular adenovirus type 12 antigens by indirect immunoferritin technique, *Int. J. Cancer* **2**:85.

Levinthal, J. D., Dunnebacke, T. H., and Williams, R. C., 1969, Study of poliovirus infection of human and monkey cells by indirect immunoferritin technique, *Virology* **39**:211.

Lohmeyer, J., Talens, L. T., and Klenk, H. D., 1979, Biosynthesis of the influenza virus envelope in abortive infection, *J. Gen. Virol.* **42**:73.

Luft, J. H., 1956, Permanganate—A new fixative for electron microscopy, *J. Biophys. Biochem. Cytol.* **2**:799.

Luft, J. H., 1959, The use of acrolein as a fixative for light and electron microcopy, *Anat. Res.* **133**:305.

Luft, J. H., 1961, Improvements in epoxy resin embedding methods, *J. Biophys. Biochem. Cytol.* **9**:409.

Lwoff, A., and Tournier, P., 1966, The classification of viruses, *Annu. Rev. Microbiol.* **20**:45.

Mandel, B., 1962, Early stages of virus–cell interaction as studied by using antibody, *Cold Spring Harbor Symp. Quant. Biol.* **27**:123.

Maraldi, N. M., 1976, Electron autoradiography of free specimens, in: *Principles and Techniques of Electron Microscopy*, Vol. 6 (M. A. Hayat, ed.), p. 271, Van Nostrand Reinhold, New York.

Markham, R., Frey, S., and Hills, G. J., 1963, Methods for the enhancement of image detail and accentuation of structure in electron microscopy, *Virology* **20**:88.

Masker, W. E., Kuemmerle, N. B., and Allison, D. P., 1978, *In vitro* packaging of bacteriophage T7 DNA synthesized *in vitro*, *J. Virol.* **27**:149.

Maul, G. G., Rovera, G., Vorbrodt, A., and Abramczuk, J., 1978, Membrane fusion as a mechanism of simian virus 40 entry into different cellular compartments, *J. Virol.* **28**:936.

McCombs, R. M., Benyesh-Melnick, M., and Brunschwig, J. P., 1968, The use of millipore filters in ultrastructural studies of cell cultures and viruses, *J. Cell Biol.* **36**:231.

Miller, M. F. II, 1974, Particle counting of viruses, in: *Principles and Techniques of Electron Microscopy*, Vol. 4 (M. A. Hayat, ed.), p. 89, Van Nostrand Reinhold, New York.

Mollenhauer, H. H., 1964, Plastic embedding mixtures for use in electron microscopy, *Stain Technol.* **39**:111.

Moor, H., Muhlethaler, K., Waldner, H., and Frey-Wyssling, A., 1961, A new freezing ultramicrotome, *J. Biophys. Biochem. Cytol.* **10**:1.

Nelson, B. K., and Flaxman, B. A., 1972, *In situ* embedding and vertical sectioning for electron microscopy of tissue cultures grown on plastic petri dishes, *Stain Technol.* **47**:261.

Nermut, M. V., 1972, Negative staining of viruses, *J. Microsc.* **96**:351.

Nermut, M. V., 1977, Freeze-drying for electron microscopy, in: *Principles and Techniques of Electron Microscopy*, Vol. 7 (M. A. Hayat, ed.), p. 79, Van Nostrand Reinhold, New York.

Newman, S. B., Borysko, E., and Swerdlow, M. J., 1949, Ultramicrotomy by a new method, *J. Res. Natl. Bur. Stand.* **43**:183.

Nomura, M., and Benzer, S., 1961, The nature of the "deletion" mutants in the rII region of phage T4, *J. Mol. Biol.* **3**:684.

Palade, G. E., 1952, A study of fixation for electron microscopy, *J. Exp. Med.* **95**:285.

Pease, D. C., 1964, *Histological Techniques for Electron Microscopy*, Academic Press, New York.

Porter, K. R., and Blum, J., 1953, A study in microtomy for electron microscopy, *Anat. Rec.* **117**:685.

Porter, K. R. and Kallman, F., 1953, The properties and effects of osmium tetroxide as a tissue fixative with special reference to its use for electron microscopy, *Exp. Cell Res.* **4**:127.

Porter, K. R., Claude, A., and Fullam, E. F., 1945, A study of tissue culture cells by electron microscopy, methods and preliminary observations, *J. Exp. Med.* **81**:233.

Poteete, A. R., and King, J., 1977, Functions of two new genes in *Salmonella* phage P22 assembly, *Virology* **76**:725.

Price, W. C., William, R. C., and Wyckoff, R. W. G., 1945, The electron micrography of crystalline plant viruses, *Science* **102**:277.

Provisional Committee for Nomenclature of Viruses, 1966, Suggestions for the classification of oncogenic RNA viruses, *J. Natl. Cancer Inst.* **37**:395.

Rassart, E., Spencer, J. H., and Zollinger, M., 1979, Localization of *Escherichia coli* RNA polymerase binding sites on bacteriophage S13 and ϕX174 DNAs by electron microscopy, *J. Virol.* **29**:179.

Reissig, M., and Orrell, S. A., 1970, A technique for the electron microscopy of protein-free particle suspensions by the negative staining method, *J. Ultrastruct. Res.* **32**:107.

Richards, K. E., and Williams, R. C., 1976, Assembly of tobacco mosaic virus *in vitro*,

in: *Comprehensive Virology*, Vol. 6 (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 1–37, Plenum Press, New York.

Richards, K. E., Williams, R. C. and Calendar, R., 1973, Mode of DNA packing within bacteriophage heads, *J. Mol. Biol.* **78**:255.

Robertson, J. D., 1964, Unit membranes: A review with recent new studies of experimental alterations and a new subunit structure of synaptic membranes, in: *Cellular Membranes in Development* (M. Lucke, ed.), Academic Press, New York.

Roeder, G. S., and Sadowski, P. D., 1977, Bacteriophage T7 morphogenesis: Phage-related particles in cells infected with wild-type and mutant T7 phage, *Virology* **76**:263.

Sabatini, D. D., Bensch, K. G., and Barrnett, R. J., 1963, Cytochemistry and electron-microscopy: Preservation of cellular ultrastructure and enzymatic activity by aldehyde fixation, *J. Cell Biol.* **17**:19.

Salpeter, M. M., 1966, General area of autoradiography at the electron microscope level, in: *Methods in Cell Physiology*, Vol. 2 (D. M. Prescott, ed.), p. 229, Academic Press, New York.

Salpeter, M. M., and Bachmann, L., 1964, Autoradiography with the electron microscope. A procedure for improving resolution sensitivity and contrast, *J. Cell Biol.* **22**:469.

Salpeter, M. M., and Bachmann, L., 1972, Autoradiography, in: *Principles and Techniques of Electron Microscopy*, Vol. 2 (M. A. Hayat, ed.), p. 221, Van Nostrand Reinhold, New York.

Sharpe, D. G., 1949, Enumeration of virus particles by electron micrography, *Proc. Soc. Exp. Biol. Med.* **70**:54.

Sharpe, D. G., 1965, Quantitative use of the electron microscope in virus research. Methods and recent results of particle counting, *Lab. Invest.* **14**:831.

Sharpe, D. G., and Beard, J. W., 1952, Counts of virus particles by sedimentation on agar and electron microgrophy, *Proc. Soc. Exp. Biol. Med.* **81**:75.

Sharpe, D. G., and Buckingham, M. J., 1956, Electron microscopic measure of virus particle dispersion in suspension, *Biochim. Biophys. Acta* **19**:13.

Sharpe, D. G., and Overman, J. R., 1958, Enumeration of vaccinia virus particles in crude extracts of infected tissues by electron microscopy, *Proc. Soc. Exp. Biol. Med.* **99**:409.

Singer, S. J., 1959, Preparation of an electron-dense antibody conjugate, *Nature (London)* **183**:1523.

Sjostrand, F. S., 1953, A new microtome for ultrathin sectioning for high resolution electron microscopy, *Experientia* **9**:114.

Sjostrand, F. S., 1967, *Electron Microscopy of Cells and Tissues*, Vol. I: *Instrumentation and Techniques*, Academic Press, New York.

Smith, H. O., 1979, Nucleotide sequence specificity of restriction endonucleases, *Science* **205**:455.

Spencer, J. H., 1979, Promoters in small bacteriophage genomes, *T.I.B.S.* **4**:164.

Stanley, W. M., 1935, Isolation of a crystalline protein possessing the properties of tobacco mosaic virus, *Science* **81**:644.

Steere, R. L., 1957, Electron microscopy of structural detail in frozen biological specimens, *J. Biophys. Biochem. Cytol.* **3**:45.

Steere, R. L., 1969, Freeze etching simplified, *Cryobiology* **5**:306.

Steere, R. L., and Erbe, E. F., 1979, Complementary freeze-fracture, freeze-etch specimens, *J. Microsc.* **117**:211.

Stevens, A. R., 1966, High resolution autoradiography, in: *Methods in Cell Physiology*, Vol. 2 (D. M. Prescott, ed.), p. 255, Academic Press, New York.

Stubbs, G., Warren, S., and Holmes, K., 1977, Structure of RNA and RNA binding site in TMV from 4 Å map calculated from X-ray fibre diagrams, *Nature (London)* **267**:216.

Stüber, D., and Bujard, 1977, Electron microscopy of DNA: Determination of absolute molecular weights and linear density, *Mol. Gen. Genet.* **154**:299.

Thach, R. E., and Thach, S. S., 1971, Damage to biological samples caused by the electron beam during electron microscopy, *Biophys. J.* **11**:204.

Thach, S. S. and Thach, R. E., 1973, Mechanism of viral replication. I. Structure of replication complexes of R17 bacteriophage, *J. Mol. Biol.* **81**:367.

Thomas, J. O., 1974, Chemical linkage of the tail to the right-hand end of bacteriophage lambda DNA, *J. Mol. Biol.* **87**:1.

Thomas, M., White, R. L., and Davis, R. W., 1976, Hybridization of RNA to double-stranded DNA: Formation of R-loops, *Proc. Natl. Acad. Sci. USA* **73**:2294.

Thompson, B. J., Escarmis, C., Parker, B., Slater, W. C., Doniger, J., Tessman, I., and Warner, R. C., 1975, Figure-8 configuration of dimers of S13 and ϕX174 replicative form DNA, *J. Mol. Biol.* **91**:409.

Thompson, B. J., Camien, M. N., and Warner, R. C., 1976, Kinetics of branch migration in double-stranded DNA, *Proc. Natl. Acad. Sci. USA* **73**:2299.

Valentine, R. C., and Horne, R. W., 1962, An assessment of negative staining techniques for revealing ultrastructure, in: *The Interpretation of Ultrastructure* (R. J. C. Harris, ed.), p. 263, Academic Press, New York.

Valenzuela, M., and Inman, R. B., 1975, Visualization of a novel junction in bacteriophage λ DNA, *Proc. Natl. Acad. Sci. USA* **72**:3024.

Vollenweider, H. J., Sogo, J. M., and Koller, T. H., 1975, A routine method for protein-free spreading of double- and single-stranded nucleic acid molecules, *Proc. Natl. Acad. Sci. USA* **72**:83.

Vollenweider, H. J., James, A., and Szybalski, W., 1978, Discrete length classes of DNA depend on mode of dehydration, *Proc. Natl. Acad. Sci. USA* **75**:710.

Vollenweider, H. J., Fiandt, M., and Szybalski, W., 1979, A relationship between DNA helix stability and recognition sites for RNA polymerase, *Science* **205**:508.

Watson, D. H., 1962, Electron-micrographic particle counts of phosphotungstate-sprayed virus, *Biochim. Biophys. Acta* **61**:321.

Watson, J. H. L., 1947, An effect of electron bombardment upon carbon black, *J. Appl. Phys.* **18**:153.

Westmoreland, B. C., Szybalski, W., and Ris, H., 1969, Mapping of deletions and substitutions in heteroduplex DNA molecules of bacteriophage λ by electron microscopy, *Science* **163**:1343.

Williams, R. C., 1953*a*, A method of freeze-drying for electron microscopy, *Exp. Cell Res.* **4**:188.

Williams, R. C., 1953*b*, The shapes and sizes of purified viruses as determined by electron microscopy, *Cold Spring Harbor Symp. Quant. Biol.* **18**:185.

Williams, R. C., 1977, Use of polylysine for adsorption of nucleic acids and enzymes to electron microscope specimen films, *Proc. Natl. Acad. Sci. USA* **74**:2311.

Williams, R. C., and Chamberlin, M. J., 1977, Electron microscope studies of transient complexes formed between *Escherichia coli* RNA polymerase holoenzyme and T7 DNA, *Proc. Natl. Acad. Sci. USA* **74**:3740.

Williams, R. C., and Fisher, H. W., 1970, Electron microscopy of tobacco mosaic virus under conditions of minimal beam exposure, *J. Mol. Biol.* **52**:121.

Williams, R. C., and Fisher, H. W., 1974, *An Electron Micrographic Atlas of Viruses*, 128 pp., Thomas, Springfield, Ill.

Williams, R. C., and Glaeser, R. M., 1972, Ultrathin carbon support films for electron microscopy, *Science* **175**:1000

Williams, R. C., and Smith, K. M., 1958, The polyhedral form of the *Tipula* iridescent virus, *Biochim. Biophys. Acta* **28**:464.

Williams, R. C., and Wyckoff, R. W. G., 1944, The thickness of electron microscopic objects, *J. Appl. Phys.* **15**:712.

Williams, R. C., and Wyckoff, R. W. G., 1945, Electron shadow-micrography of virus particles, *Proc. Soc. Exp. Biol. Med.* **58**:265.

Williams, R. C., and Wyckoff, R. W. G., 1946, Applications of metallic shadow-casting to microscopy, *J. Appl. Phys.* **17**:23.

Williams, R. C., Kass, S. J., and Knight, C. A., 1960, Structure of Shope papilloma virus particles, *Virology* **12**:48.

Williams, R. C., and Fisher, H. W., 1979, Binding sites of *E. coli* RNA polymerase to ϕX174 DNA RF, in: *37th Annual Proceedings of the Electron Microscopy Society of America* (G. W. Bailey, ed.), p. 34, Claitor's Publishing Division, Baton Rouge.

Yeger, H., and Kalnins, V. I., 1978, Immunocytochemical localization of gp70 over virus-related submembranous densities in *ts* mutant Rauscher murine leukemia virus-infected cells at the nonpermissive temperature, *Virology* **91**:489.

Zachary, A., and Simon, L. D., 1977, Size changes among λ capsid precursors, *Virology* **81**:107.

Zagury, D., Pappas, G. D., and Marcus, P. I., 1968, Preparation of cell monolayers for combined light and electron microscopy: Staining in blocks, *J. Microsc. Paris* **7**:287.

CHAPTER 3

Structural Studies of Viruses with X-Rays and Neutrons

Bernard Jacrot

Laboratoire Européen de Biologie Moléculaire
38041 Grenoble Cédex, France

1. INTRODUCTION

Structural studies of viruses were reviewed in 1967 by Finch and Holmes (1967) and in 1972 by Finch. Those studies were based on the two available techniques: electron microscopy and X-rays. The use of X-ray diffraction to investigate virus structures from single crystals was just beginning, and although high resolution was considered as a remote possibility, only low-resolution information was then available. Electron microscopy is a very popular approach because the virologist can see an image, even if the quantitative interpretation of that image is far from simple; in fact, this technique is based on the same methods as the interpretation of X-ray diffraction. Since 1972, however, the latter method has been considerably developed, and some structures of viruses at high resolution are now available. The folding of the polypeptide chain of a few coat proteins is now known, as well as the nature of the interactions between those proteins. Another advance has been in the application of neutron scattering to virology; this technique has increased the quality and the amount of information which can be obtained from a low-resolution study, particularly from a virus solution.

The concept of organization of viruses on helical or icosahedral symmetry (Caspar and Klug, 1962) now is generally accepted. From the

work that we shall review, a clearer picture has emerged on how viral proteins are made to fulfill the requirements of those symmetries, and which part of a virus, particularly of an isometric virus, has symmetry and which part does not.

We shall not attempt to describe in detail the methods used with X-rays and neutrons but rather try to explain the basic principles and focus on what has been obtained and could be obtained by the different techniques. Details essential for those who want to apply them can be found in various textbooks and review papers on X-ray crystallography (Blundell and Johnson, 1976), X-ray small-angle scattering (Guinier and Fournet, 1955; Kratky and Pilz, 1972; Luzzati *et al.*, 1976), and neutrons (Bacon, 1975; Jacrot, 1976; Kneale *et al.*, 1977). There are, in fact, many common features between X-ray and neutron methods, and we shall try to emphasize their similarities and complementarities.

2. PHYSICAL BASIS OF X-RAY AND NEUTRON SCATTERING

When an electron, a photon, or a neutron hits an atom, it may be either absorbed or scattered. The observation of an object is really observation of the scattered waves. In the case of light or electrons, these waves are observed through optical devices such as lenses, which recombine the waves to form an image. In the case of X-ray or neutrons, one does not know how to make efficient devices to form such an image; instead, one measures the waves scattered by the object as a function of angle and, if the sample is a single crystal, of its orientation; from those sets of measurements, one tries to do by calculation what the optical systems do physically with light or electrons, namely, to construct an image of the object.

In such an object, the waves scattered by its different atoms interfere, and the waves which result from these interferences are received by the X-ray or neutron detector. Let us first consider the scattering by one virus. Each atom has a given probability of scattering the incident wave. This probability is expressed through a number, b, called scattering amplitude. For a given atom, this scattering amplitude is totally different for X-rays and neutrons (see Table 1). For X-rays, this amplitude is proportional to the number of electrons of the atom. For neutrons, the scattering is by the atomic nucleus, and there are no simple correlations between the atomic number and the scattering amplitude. Isotopes of the same atom have different scattering amplitudes. This difference is very large for hydrogen and deute-

TABLE 1

Scattering Amplitudes of Various Atoms for X-Rays and Neutrons[a]

Atom	Neutrons	X-rays
Hydrogen	−0.3742	0.28
Deuterium	0.6671	0.28
Carbon	0.6551	1.67
Nitrogen	0.940	1.97
Oxygen	0.5804	2.25
Sodium	0.36	3.10
Magnesium	0.52	3.38
Phosphorus	0.52	4.23
Sulfur	0.28	4.51
Chlorine	0.96	4.79
Potassium	0.37	5.36
Calcium	0.47	5.64

[a] Scattering amplitudes are listed in units of 10^{-12} cm. For X-rays the amplitude is for the forward scattering.

rium, and plays an essential role in the use of neutrons to study biological objects. The number b gives the relative amplitude of the wave scattered in the forward direction; thus it can be negative, whereas the scattering probability is proportional to b^2 and is always positive.

With both types of radiation, the scattering amplitude is of the same order of magnitude and leads to a very small probability for scattering by a single virus particle. The observation will therefore always be done with a large number of particles. (A similar situation exists with electron microscopy: Individual virus particles are observable only if stained or shadowed. Unstained viruses can be observed only when they form a crystalline array; then the use of electron microscopy is quite similar to diffraction techniques with X-rays.)

There are two types of samples with a large number of viruses: solutions in which the viruses have random orientations and samples with correlations between orientations of the virions. In the first case (scattering by solutions), because of the random orientation, the amount of structural information is limited. With such a specimen, the diffraction is usually referred to as "small-angle scattering." (The sample diffracts at all angles, but most of the intensity is concentrated at small angles, and only that part is useful.) Icosahedral viruses are nearly spherical, so that there is less information lost from the random orientation, and more structural details can be obtained from small-angle scattering than in the case of viruses of other shapes.

Samples with correlations between orientations are of a different nature for spherical viruses and elongated viruses. For spherical viruses, one uses a single crystal. Such a crystal is made up of identical particles on a regular lattice. Each virus can be correlated with another one by simple symmetry relations. In that case, if the virus particles are really identical and in nearly perfect order, detailed structural information can be obtained, which in the ideal case would be the localization of all atoms within the virus. The method is an extension of that used in protein crystallography and is referred to as "single crystal diffraction." With elongated viruses, one uses an oriented gel. In such a sample, all the virus rods are very nearly parallel, and, if the analysis cannot be brought to atomic scale, it can be pushed to a stage where the folding of the polypeptid chain is observable. This case is referred to as gel diffraction.

We shall not give detailed descriptions of those methods, which go beyond the scope of this chapter but rather give the general physical and mathematical background applicable to each case. This chapter is not intended to be a reference paper for virologists who wish to do structural analysis but rather a critical review of existing results, so that for each method the emphasis will be put on its intrinsic limitations. For detailed descriptions, the reader will be referred to the appropriate literature.

The virus must be considered as a distribution of scattering matter $\rho(\vec{r})$. ρ is the density of scattering amplitude at the point of coordinate $\vec{r}$ inside the virus. The scattered waves can be expressed in terms of this distribution. (For a derivation, see, for instance, Guinier, 1963.) An elegant explanation is given in Finch and Holmes (1967) by

$$F(\vec{s}) = \int_{-\infty}^{+\infty} \rho(\vec{r}) \exp(2\pi i \vec{r}\vec{s})\, d^3\vec{r} \tag{1}$$

where $\vec{s}$ is a vector which defines the direction of observation, as shown in Fig. 1. Its length is given by

$$s = \frac{2}{\lambda} \sin\theta \tag{2}$$

where λ is the wavelength of the incident radiation (between 0.1 and 0.2 nm for X-rays, between 0.1 and 2 nm for neutrons). 2θ is the scattering angle, and $F(\vec{s})$ is a complex number

$$F(\vec{s}) = A(\cos\psi + i \sin\psi) \tag{3}$$

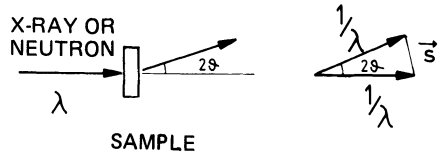


Fig. 1. Definition of the scattering vector $\vec{s}$. λ is the wavelength of the incident beam, typically, 1.5 Å for X-rays and between 1 and 20 Å for neutrons.

where A is the amplitude and ψ the phase of the scattered wave in the direction $\vec{s}$. This use of a complex number to represent a wave is very useful because the sum of the waves is mathematically expressed as the sum of those complex numbers. The mathematical relation (1) between $\rho(\vec{r})$ and $F(\vec{s})$ is known as a Fourier transform. An important property of this relation is that it can be inverted to give

$$\rho(\vec{r}) = \int_{-\infty}^{+\infty} F(\vec{s}) \exp(-2\pi i \vec{r}\,\vec{s})\, d^3r \tag{4}$$

Experimentally, one receives the X-rays or the neutrons on a detector or a photographic plate that will measure an intensity which is $[A(\vec{s})]^2$. The phase angle θ is not directly measured, and expression (4) cannot be used directly to get $\rho(\vec{r})$, which is the structure of the virus. As we have already stated, the intensity scattered by a single virus would be too small to be observable, and one must use either a solution, a single crystal, or an oriented gel. We shall now consider those various cases.

2.1. Single Crystal Diffraction

In a single crystal, the viruses are closely packed and are arranged periodically. Interferences will arise from atoms of neighboring viruses as well as from atoms within a virus, so that $\rho(\vec{r})$ will be not the distribution of scattering matter within a virus but rather the distribution of matter within the crystal. Because this distribution is periodic, it can be divided in two terms: the distribution within a virus and the distribution of the viruses in a regular lattice. Thus one can write

$$\rho(\vec{r}) = \rho(\vec{r})_{\text{virus}} * \rho_{\text{lattice}} \tag{5}$$

which represents the mathematical expression for the result of the product of two distributions, called product of convolution. The Fourier

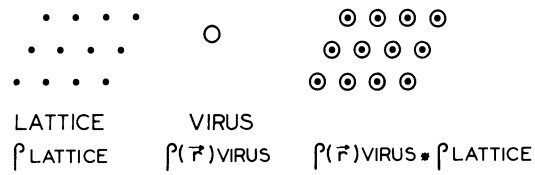


Fig. 2. Distribution of matter in a single crystal as the product of two distributions. In reality, the lattice is in three dimensions; there may be more than one virus in each repetitive unit, and they are not necessarily on the points of the lattice.

transform of such a product is the product of the Fourier transforms of the two distributions. The regular lattice is a set of points (Fig. 2) whose Fourier transform is another set of points, also forming a regular lattice which is called the reciprocal lattice.

This means that the scattered wave $F(\vec{s})$ will be zero everywhere except at points $\vec{s}$, where the transform of the virus is passing through one of those points of the reciprocal lattice. In a diffraction experiment with a single crystal, the scattered intensity will be concentrated in a well-defined direction, and the detector or the photographic plate will record a set of spots called Bragg spots (Fig. 3). This set of spots will vary if one changes the orientation of the crystal relative to the incident beam. A diffraction experiment consists of recording these spots and measuring their intensity. The various strategies used to do so are described in detail in Blundell and Johnson (1976). From those data, the structure of the virus must be derived using relation (4), which is still valid with the exception that $F(\vec{s})$ will be nonvanishing only for

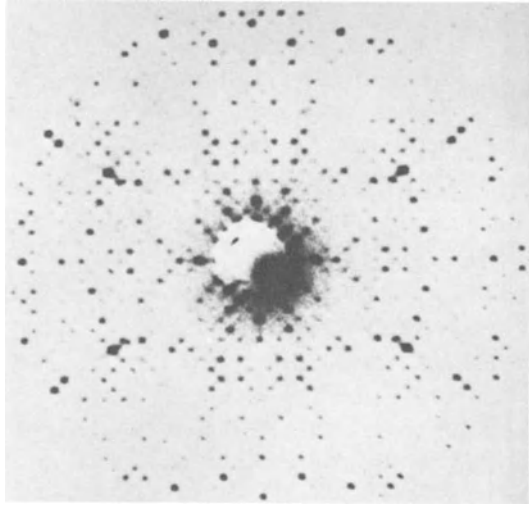


Fig. 3. X-ray diffraction pattern obtained with tomato bushy stunt virus (from Harrison, 1969). These are the low-resolution data, which have been used in particular to establish the icosahedral symmetry of the virus and the orientation of the isosahedron in the crystal lattice (see Fig. 4).

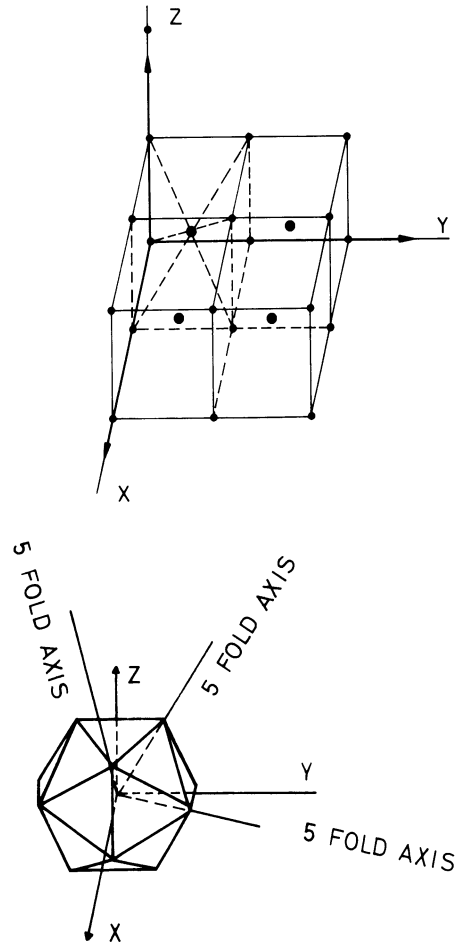


Fig. 4. Crystal lattice and virus icosahedron. The top part is a representation of the crystal lattice form in a single crystal of TBSV. A virion is sitting on each of the dots. The bottom part is an icosahedron representation of the virion on the same scale and with the same orientation with respect to the crystal lattice. The virion has the same symmetry as an icosahedron. Only some of the 5-fold axes are shown; one sees that those axes are different from the axis of symmetry of the lattice. This figure also suggests the dense packing of the virions in the crystal.

well-defined values of s; thus the integral in (4) can be replaced by a summation. However, we are left with two difficulties which are the main problems in protein crystallography.

1. The detector measures an intensity $[F(\vec{s})]^2$ and so gives no information on the phase angle ψ [see relation (3)], and relation (4) can be used only if $F(s)$ is known, with its amplitude and its phase. This difficulty is known as the phase problem.

2. Data are collected only up to a maximum value of the length of the scattering vector, and the application of relation (4) with a limited set of data leads to a deformation of $\rho(\vec{r})$. One says that the data have been collected up to a certain resolution, which is defined by

$$\text{resolution} = \frac{1}{s_{\text{maximum}}}$$

2.1.1. The Resolution Problem

The better the resolution, the easier is the interpretation of the map $\rho(\vec{r})$. With a resolution of 2 Å, each amino acid and each nucleotide is localized with accuracy; at 5 Å, the distinction between protein and nucleic acid is difficult. At 10 Å and lower resolution, only the gross organization of the virus and its symmetry are revealed.

There are several factors which limit resolution. Some are instrumental and can be improved with technical development; others are more fundamental. The main experimental factors are as follows:

1. The wavelength used for the experiment. The resolution is at best $\lambda/2$. This is not a limitation for X-ray; however, it may be limiting if one uses neutrons of long wavelength.

2. The amount of data to collect increases as s^3, and the time to collect them increases even more. In the case of tomato bushy stunt virus, the data at a resolution of 2.85 Å include 198,000 reflections (Harrison *et al.*, 1978). Also, when collecting data to high resolution, there are practical problems in separating the spots, which are very close. These problems, although difficult, can be solved by technical developments: use of a large detectors to collect simultaneously a large number of data with a high efficiency, or use of very intense X-ray sources such as synchrotron radiation (Stuhrmann, 1978).

The much more fundamental limitation to resolution is the fact that a crystal diffracts only up to some resolution. It is a well-known fact that intensities diffracted by a protein on a virus fade out much more quickly than they would if crystals were perfect. This is due to disorder in the crystals, which may have several origins. Protein or virus crystals are highly hydrated, and physical contacts between neighboring particles are limited. This may lead to some imperfections in the packaging. Another factor is that a virus is not a rigid structure, and part of it may oscillate around an average position. This also introduces differences between viruses on the lattices, and it limits the resolution.

The above points apply to proteins as well as to viruses. However, there is a last point which is specific to viruses. The viruses that have been crystallized are all icosahedral. The outer parts of these viruses are well ordered and highly symmetrical. The packaging in the crystal is due to interactions between these parts and is rather good. The possibility of crystallizing biological samples is related to the availability of large quantities of purified material. As viruses are usually well purified, many of them have been easily crystallized. This applies to plant and animal viruses; no crystallization of bacteriophage has yet

been reported. In infected cells, viruses are often well ordered and form a genuine single crystal (see, for instance, Henry *et al.*, 1971). But in the interior of the virion, the situation may be different. This is well illustrated by the case of cowpea chlorotic mottle virus (CCMV). This virus is easily crystallized (Rossmann *et al.*, 1973). However, CCMV has a divided genome with four components (Fowlks and Young, 1970) of molecular weights 1.0, 1.1, 0.8, and 0.3 million packaged in three different virions. The interior of a virion with a strand of RNA of 1 million and that of one which has two stands of 0.8 and 0.3 million cannot be identical. Thus, in such a crystal, even if the outer part of the capsid is perfectly ordered, the interior of the virions can form only a highly disordered crystal, or, in fact, it may have no order at all. This point will be discussed again later when reviewing the experimental results.

2.1.2. The Phase Problem

The phase problem has been solved in X-ray protein crystallography by the method of isomorphous replacement (Green *et al.*, 1954). In this method, a heavy atom, which scatters strongly, is introduced in the crystal. If the atom is bound to the protein in a well-defined position, the intensities of Bragg reflections are modified. In the hypothesis that the protein is not deformed (isomorphous) by that atom, the measurement of these modifications of intensity allows a determination of the localization of the heavy atom, and then of the phases of each reflection. An ambiguity remains which is removed by a similar experiment done with another heavy atom derivative. This method applies to virus crystallography and is used [see references later in the review of the results obtained with tomato bushy stunt virus (TBSV), southern bean mottle virus and satellite tobacco necrosis virus].

In the virus crystal, there is in addition to the crystal symmetry (which correlates the various virions in the crystal) the icosahedral symmetry of the virions (which correlates various parts of the virion). This provides complementary methods which transform an otherwise practically impossible task (the phasing of several hundred thousand reflections by isomorphous replacement) to a difficult but feasible one. A first use of icosahedral symmetry is in the treatment of the picture obtained by negative staining in electron microscopy (see the chapter on electron microscopy). That treatment provides rather precise information on the outer shape of the virion. This provides some phase informa-

tion, as all previous structural knowledge of the virus will impose constraints on the phases. This has been used at low resolutions for TMV disks (Jack, 1973) and for TBSV (Jack *et al.*, 1975). However, the icosahedral symmetry imposes many more constrains. For instance, the fivefold axis correlates subunits within each virion, and those correlations put restrictions on the phases of the various reflections. In other terms, there are identical parts of the virions which are in different positions in the unit cell, related by noncrystallographic symmetries, and these will provide redundant information. Practical ways of using these redundancies have been worked out by Bricogne (1976). These methods are very powerful and have provided a structure of tomato bushy stunt virus at 2.9 Å resolution (Harrison *et al.*, 1978). Similarly, the use of the 17-fold axis present in disks of TMV proteins has helped to solve the structure of that protein (Bloomer *et al.*, 1978).

This method has an intrinsic limitation: the structural analysis is done by supposing the existence of the icosahedral symmetry, so that the parts of the virion which do not follow that symmetry will not be analyzed. However, it is likely that this is not a further limitation than that imposed by single crystal analysis mentioned above. Let us consider two virions in the crystal. Because the exterior, as far as one knows, follows strict icosahedral symmetry, those virions can be in any of the orientations around, for instance, their 5-fold axis. If all the parts of the virion have the icosahedral symmetry, then those two virions will be perfectly ordered in the single crystal. But if part of each of the virions does not follow that symmetry, that part will not be ordered inside the crystal, and will not contribute to the diffraction pattern. Supposing icosahedral symmetry in analyzing the diffraction is therefore not introducing further limitations. However, one cannot completely exclude that the exterior of the virus is sensitive to its internal organization, and that very subtle departures from strict icosahedral symmetry will impose an orientation of the virions inside the crystal which will make it ordered even for the nonicosahedral central part. However, this is very unlikely.

2.1.3. Neutron Diffraction

All the general physical arguments about diffraction apply to both X-ray and neutron diffraction. However, the solution of the phase problem by isomorphous replacement makes use of the fact that heavy atoms scatter X-rays much more strongly than the light atoms which

compose the virus. Table 1 shows that there is no correlation for neutrons between the weight of atoms and their scattering power, therefore, this method is inapplicable with neutrons. But, in fact, because neutrons are less commonly available than X-rays, neutron diffraction is a technique used after X-ray diffraction has been used up to its limits. Such a limit does exist, as just explained, due to deviation from icosahedral symmetry. Deviation from icosahedral symmetry is likely to be gradual. If part of a viral protein follows strict icosahedral symmetry, another part a few angstroms away cannot depart very strongly from that symmetry. At low resolution, one expects that a much larger part of the virion will follow that symmetry. Single crystal diffraction at low resolution should thus provide information on the structure of parts of the virus which cannot be seen on a high-resolution diffraction map. The case of TBSV (Harrison *et al.*, 1978; Chauvin *et al.*, 1978*b*) suggests that departures from icosahedral symmetry appear where the protein and the viral RNA interact. A low-resolution electron density map of that region is impossible to interpret because the density of protein and hydrated RNA are quite similar. It is at that stage that neutron diffraction finds its usefulness. At low resolution (10 Å and above), the apparent scattering density of proteins and nucleic acid is reduced because of the water of hydration. This is why, for X-rays, protein and nucleic acid are indistinguishable at low resolutions.

The situation is totally different with neutrons. Table 2 shows that H and D atoms have very different scattering amplitude. As a consequence, H_2O and D_2O have very different scattering densities. One can also define and calculate scattering densities for proteins and RNA. Those densities are somewhat a function of the amino acid compositions, but, practically, not of base compositions. Data for those calculations can be found in Jacrot (1976). Figure 5 shows the scattering densities calculated for water, a viral protein from brome mosaic virus (BMV), and viral RNA, as a function of the percentage of D_2O in the water. The variation of the scattering density of the protein and RNA with the amount of D_2O (in the solvent or the water of crystallization) is due to the exchange of labile protons for deuterium atoms. Those calculations show that, within the limit of low-resolution crystallography, the contribution to the scattering density map of RNA and protein will depend on the amount of D_2O in the water of crystallization. To a good approximation, with 42% D_2O, the protein will not contribute to the map, whereas with about 68% D_2O, it will be the RNA that does not contribute, and maps obtained with various percentage of D_2O will be easier to interprete than low-resolution X-ray maps. However, to obtain those neutron maps, the phase problem must be solved. Because the

TABLE 2

Scattering Densities of Various Compounds[a]

	Neutrons	X-rays
H_2O	−0.562	9.40
D_2O	6.404	9.40
Protein in H_2O	~1.8	~12
Protein in D_2O	~3.1	~12
RNA in H_2O	3.54	15.7
RNA in D_2O	4.55	15.7
DNA in H_2O	3.54	15
DNA in D_2O	4.29	15

[a] Densities for neutrons and X-rays are in 10^{-14} cm/Å^3. For X-rays the correspondence with the more usual unit of electron per Å^3 is 10^{-14} cm/Å^3 ≡ 0.0356e/Å^3. For proteins and nucleic acids the scattering density values are dependent on the value choosen for the specific volume. The values have been calculated for a specific volume of nucleic acids of 0.55 cm^3/g. For proteins the scattering density is also somewhat dependent on the amino acid composition. Typical values of scattering densities of solvent used for contrast variation with X-rays are as follows: a 55% sucrose solution has a scattering density of 11.6 10^{-14} cm/Å^3 (0.412e/Å^3), close to the protein scattering density; a 5 M solution of NaBr has a scattering density of 13.310^{-14} cm/Å^3 (0.475e/Å^3), smaller than that of a nucleic acid.

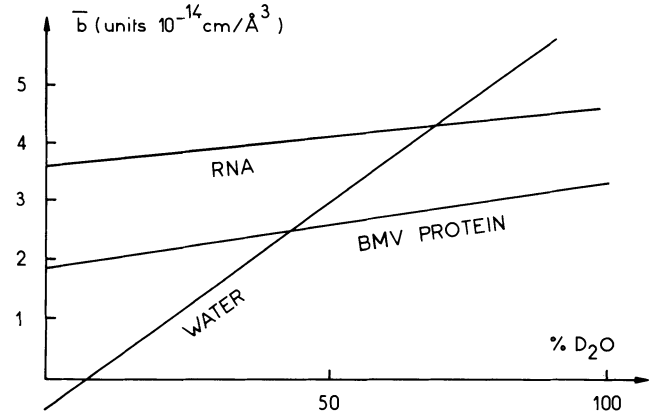


Fig. 5. Scattering densities of water, protein, and RNA. This figure is relevant to neutron scattering. See also Table 2.

outer shape of the virus is known from previous work with electron microscopy and X-rays, that information could be used for phase determination following Jack *et al.* (1975). One can use the X-ray phases for the crystal with no D_2O, and then use the shape of the virus to deduce the phases in the crystals with various amounts of D_2O in the same way as a heavy atom derivative.

2.2. Oriented Gels

Long, helicoidal viruses can be packed in oriented gels in which all viruses are nearly parallel to each other but with no correlation between their center of gravity. This can be done with TMV (Bernal and Fankuchen, 1941) and with filamentous bacteriophages such as fd or Pf1 (Marvin, 1966). Figure 6 shows an experimental diffraction pattern obtained with a TMV gel.

At first sight, this pattern shows some similarity to that obtained with a single crystal as shown in Fig. 3. In fact, however, those patterns

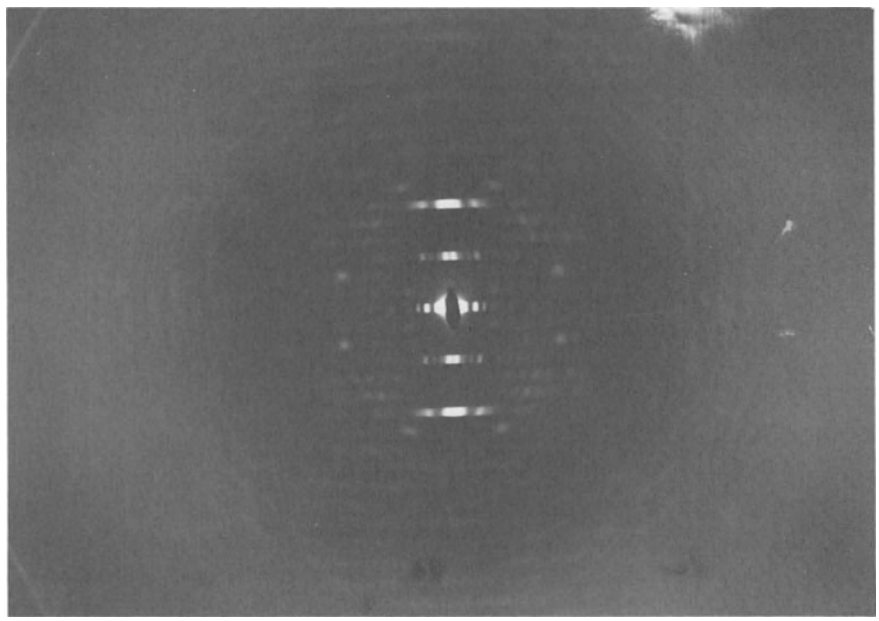


Fig. 6. Fiber diffraction pattern from an oriented gel of TMV. Photograph taken by Dr. K. Holmes. Notice the layer structure. Every third line has strong intensity on or near the vertical axis (meridional). They correspond to the pitch of the helix (23 Å).

are formed in very different ways: with a single crystal, spots are formed at positions defined by the crystal lattice (and not by the structure of the virus, which will affect only the intensities). Here the pattern is given by individual viruses, and it reflects directly the structure of the virus and not its packaging, which is irrelevant except for the parallelism of the rods. Quite essential to the theory of the formation of this pattern is the helicoidal symmetry of the virus. This theory has been developed by Cochran *et al.* (1952) and Klug *et al.* (1958). The intensities diffracted by a helicoidal object are concentrated on equidistant layer lines perpendicular to the axis of the helix (see Fig. 7). The distance between those lines is inversely proportional to the pitch of the helix. This point will be discussed later, with the results. The distribution of the intensity within those lines is a function of the matter within the elementary unit, which, after application of the helicoïdal symmetry, makes the virus. The mathematical relations between the density of scattering material and the intensities within lines are somewhat similar to relation (1), but are more complex, and involve

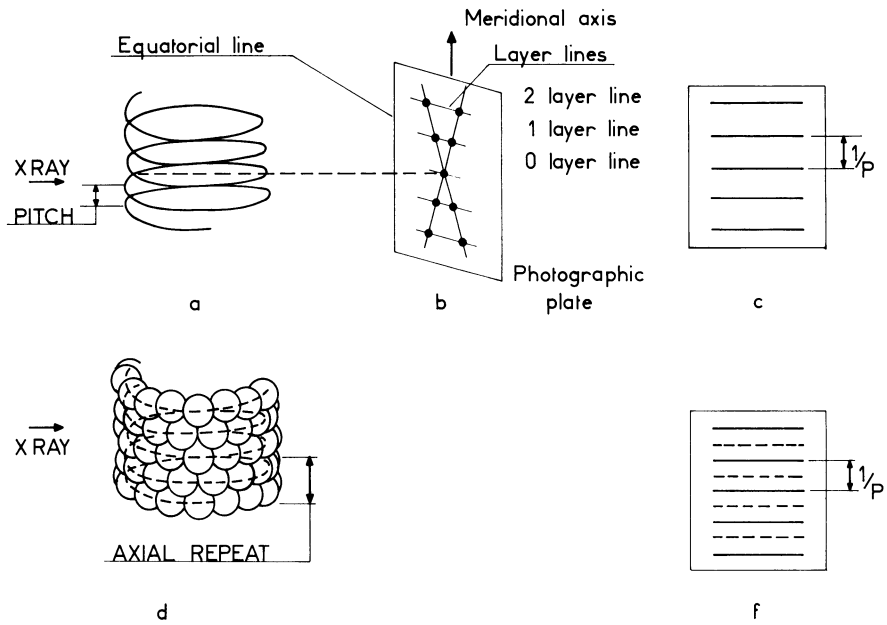


Fig. 7. Diffraction of X-rays by helices and helical viruses. The top part shows the diffraction by a simple helix. The bottom part is by a helical virus in which subunits (shown as spheres) are on identical positions of the helix only after two turns. In this case, the layer lines of the diffraction pattern are two times closer than those from a simple helix.

mathematical functions known as Bessel functions. (Those expressions are given in Finch and Holmes, 1967.) For a gel, the experimental intensities are those obtained after cylindrical averaging around the axis of the helix. Those intensities vary continuously in each line, with a series of maxima and minima which are consequences of the properties of the Bessel function. The inversion of the data to get the structure of the virus is done by methods which are extensions of those used with a single crystal, such as phasing with isomorphous heavy atom derivatives. However, as pointed out by Holmes *et al.* (1975), there are other difficulties with those gels, which arise mostly from the cylindrical averaging, and there is little hope that very high resolution will ever be obtained from gels. The 4 Å map obtained by Stubbs *et al.* (1977) for TMV may very well be the best one can expect from a very well-oriented gel.

2.3. Virus Solutions

In a solution, there is no correlation between the orientations of the viruses, and the intensities recorded are those calculated from relation (1) after averaging overall orientations of the virus.

It is clear that, in this averaging process, most of the structural information is lost. In other terms, a virus is a three-dimensional object, and, to get its structure, three-dimensional information is needed. In single-crystal diffraction, this is achieved by recording a two-dimensional diffraction pattern as a function of crystal orientation. Here, the only experimentally observable quantity is $I(s)$, which, as the consequence of the averaging, is symmetrical around the incident beam and depends only on the length of s ($\sin \theta/\lambda$, where 2θ is the scattering angle). This is illustrated in Fig. 8. We have just one-dimensional information.

2.3.1. Case of Icosahedral Isometric Viruses

If we were considering a perfectly spherical virus, which would be characterized only by a radial distribution of matter $\rho(R)$, also a one-dimensional quantity, then it would be possible to deduce this $\rho(R)$ from the measured quantity $I(s)$. In fact, one shows that

$$F(s) = \frac{2}{s} \int_0^\infty r\rho(r) \sin 2\pi rs dr \tag{6}$$

$$\rho(r) = \frac{2}{r} \int_0^\infty sF(s) \sin 2\pi rsds \tag{7}$$

$F(s)$ as shown by relation (7) is now a real number and the experimental intensity is related to $F(s)$ by

$$I(s) = |F(s)|^2 \tag{8}$$

In such a case, the radial distribution $\rho(r)$ would be easily obtained from the experimental values of $I(s)$. The only ambiguity would be in the sign of $F(s)$. But, from relation (7), it is clear that $F(s)$ must be a well-behaved function, with oscillating signs, as shown in Fig. 8. However, an icosahedral isometric virus is not a spherical object, and the knowledge of $\rho(R)$ is not easily interpreted in term of virus structure. We shall now discuss these two points.

That an icosahedral isometric virus is not a spherical object has been discussed by Harrison (1969) and Jack and Harrison (1975). Nonspherical contributions to the scattering curve become significant for a scattering vector which corresponds roughly to subunit distances which are typically 40 Å. Those contributions have been calculated for various types of organizations of the subunits within the icosahedral symmetry (e.g., dimer clustering, hexamer-pentamer clustering).

In practice, it seems difficult to use these nonspherical contributions to characterize the type of clustering. However, they will limit the

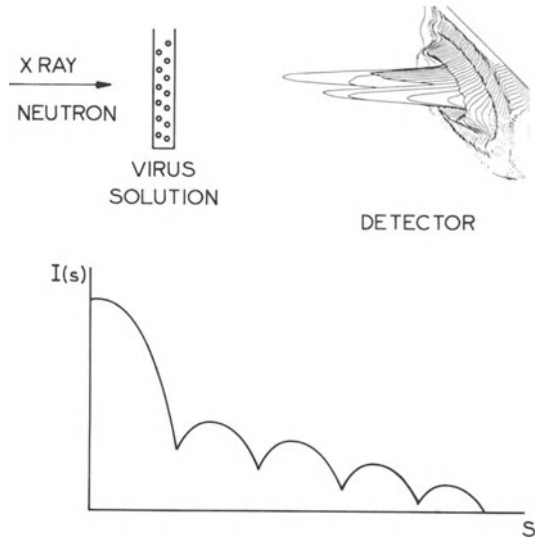


Fig. 8. X-ray and neutron scattering from solutions. For spherical viruses, the intensity is distributed on annular rings around the incident beam. The distribution of the intensity vs. the scattering vector is shown in the bottom part. The intensity at the origin is proportional to the molecular weight of the virion (for a given concentration) and can therefore be used to measure it.

domain of scattering which can be analyzed by spherical approximation, and which allows the use of relations (6) and (7). As this domain is limited to about 40 $Å^{-1}$, it will consist, for small plant viruses (radius of 150 Å), of only three or four maxima, whereas for a much larger virus the spherical approximation will be valid for a much larger number of maxima.

The second point, the interpretation of $\rho(r)$, requires some care. First, the virus is in a solvent which has a uniform scattering density, ρ_S, and only the difference of density between the virus and the solvent is relevant. So (6) and (7) should in fact be rewritten

$$F(s) = \frac{2}{r} \int_0^\infty r[\rho(r) - \rho_s] \sin 2\pi rs dr \tag{9}$$

$$\rho(r) - \rho_S = \frac{2}{r} \int_0^\infty sF(s) \sin 2\pi rs ds \tag{10}$$

$\rho(r)$ is defined by the chemical composition and can be calculated from values of Table 1. The difference $\rho(r) - \rho_s$ is called the contrast. If there were no hydration of the components of the virus, $\rho(r)$ would be calculated from the chemical composition and the specific volumes of the nucleic acids, proteins, and possibly lipids. But with hydration, the scattering matter is spread over a larger volume, and its density is consequently reduced. For both X-rays and neutrons, the scattering density of protein and nucleic acid is larger than that of ordinary water (see Table 2), but a strong hydration will reduce this density so that the knowledge of $\rho(r) - \rho_s$ does not directly give the chemical composition at radius r. For instance, with X-rays, a highly hydrated nucleic acid and a protein may well give the same contrast with solvent. However, that chemical information can be obtained if the scattering density ρ_s of the solvent is varied, so that the contrast of the various components of the virus is modified. Table 2 gives typical scattering densities for both X-ray and neutron scattering, and Fig. 4 shows how those densities vary, in the case of neutrons, with various amounts of D_2O in the solvent. One sees from that table that, with both techniques, one can modify the contrast strongly. We shall analyze the consequences of those modifications for neutrons.

One sees from Table 2 and Fig. 4, that, with 40% D_2O in the solvent, the contrast of the protein with that solvent vanishes. This value of 40% is somewhat dependent on the amino acid composition and specific volume of the protein. Similarly, with 68.70% D_2O, the

contrast of RNA vanishes, and, with about 65%, it is that of DNA, which is zero. This obviously provides a basis for a chemical interpretation of $\rho(r)$. The variation of $\rho(r) - \rho_S$ with the D_2O percentage in the solvent for a virus made only of nucleic acid and protein directly gives the relative amount of those two components at each radius. For a three-component virus, this is also feasible because, at a given radius, there are usually only two components (protein and nucleic acid, or protein and lipids). The presence of some salt and buffer in the solvent does not appreciably modify the scattering density of the solvent, at least for usual molarities. For instance, neutrons will not distinguish between pure water and water with 200 mM KCl and 50 mM tris-HCl.

Similar methods of contrast variation have been used with X-rays (see, for instance, Harrison, 1969; Zipper, 1971; Luzzati *et al.* 1976). The scattering density of the solvent is varied by adding salts or sucrose (see Table 2). There are two drawbacks in the use of X-rays for contrast variation with make the use of neutrons preferable. The first is that density matching of the protein can be achieved only with a very high molarity of salt and sucrose. High molarity of salt in several cases will destabilize the virus, or at least will modify its structure (see, for instance, the case of brome mosaic virus, Chauvin *et al.*, 1978*a*). The effect of high molarity of sucrose is not well known, but the properties of water are certainly modified, and modification of the structure cannot be excluded. Also, the density matching of nucleic acid cannot be achieved, and the analysis is consequently less accurate. Another drawback is that, with high salt (or sucrose) molarity, the solvent is no longer a uniform medium. There are regions in close contact with the protein and the nucleic acids which form the so-called hydration shells. The penetration of salt and sucrose in these domains is unknown, but it is generally accepted that the molarity of sucrose, for instance, will be smaller there than in the bulk of the solvent. For instance, in small plant viruses, the RNA is highly hydrated, and, if this water of hydration is not penetrated (or is penetrated poorly) by the salt or sucrose added to the solvent, the contrast between the electron density of this RNA domain and the solvent with salt will be diminished, and the RNA may be confused with protein. It is likely that this confusion has been made in the interpretation of some X-ray scattering.

2.3.2. Method of Analysis with Spherical Viruses

There are two possible approaches to analysis of spherical viruses. The first is to use equation (9) and derive the scattering density from

the experimental data. The weak point of that approach is the already discussed limitation of the validity of the spherical approximation. Thus one either uses the experimental data beyond the limits of this approximation, which induces errors, or one applies equation (9) only to the data where this approximation is valid, and this induces spurious ripples in $\rho(r)$ which could lead to an incorrect interpretation. However, this method has the advantage that no *a priori* knowledge of the organization of the virus is necessary. If the concentration of the sample is known and the intensity of the incident beam is measured, the intensity will be on an absolute scale, and, consequently, the $\rho(r)$ at various contrasts will also be in absolute scale, giving the amount of protein, nucleic acid, lipids, and water at each radius. This will also give the total molecular weight of the various component of the virus (Jacrot and Zaccaï, 1980).

The only approach is to use equation (10), searching for a model of the virus made of successive spherical shells which will account for the experimental curves at various contrasts. The advantage of that method is that one makes full use of the data within the range where the spherical approximation is valid. Deviations from this approximation are easily identified and in some cases could yield information on the clustering of the subunits. Also, experimental smearing, always present in neutron scattering, is easily taken into account. This approach has been developed by Schneider *et al.* (1978) and Chauvin *et al.* (1978*b*).

2.3.3. The Case of Rod-Shaped Viruses

The small-angle scattering by rod-shaped objects has been discussed by Luzzati (1960). It yields two quantities: a cross-sectional radius of gyration, and a mass per unit length. In our view, there is little hope for much useful information from this radius of gyration, but the mass per unit length is very important in building a model. So far, this quantity has been measured only for bacteriophages fd and Pf1 (Torbet, private communication), but this could be useful in the large class of filamentous viruses.

2.3.4. The Case of Oddly Shaped Viruses

If a virus is neither quasi-spherical nor very elongated, no simple analysis can be made from solution scattering. However, if a minimum of information is available from electron microscopy and chemical

analysis, one could try to build a model of the virus, calculate its scattering in various contrasts, and look for a fit with experimental data. The relevant formula to calculate the scattering by an object of any shape can be found in Guinier and Fournet (1955). This method has been used for the case of alfalfa mosaic virus (S. Cusak and J. Mellema, private communication). This virus is made of several bacilliform particles made of RNA and a unique type of polypeptide chain. This model calculation approach was good enough to establish that the RNA does not penetrate through the protein shell to the surface of the virion even though the virus is ribonuclease-sensitive. This experiment was done with neutron scattering and shows the possibility of getting structural information even with a virus which does not have a simple shape.

2.4. Experimental Considerations

2.4.1. Sources

X-ray sources used so far are convenient ones, namely, X-ray generators which are available in many laboratories. A new development is the availability of synchrotron radiation as an X-ray source (Phillips, 1976; Stuhrmann, 1978). Such sources are much more intense than the conventional ones, and could be quite useful in investigations of the structure of the large viruses by X-ray diffraction.

Neutron sources are nuclear; so far, experiments on viruses have been done only at high-flux facilities such as those existing at Brookhaven (United States) or Grenoble (France), but undoubtedly experiments are possible on medium-flux reactors, which are available at several universities. Standard X-ray sources provide a monochromatic beam, usually of wavelength 1.54 Å. A neutron source provides a white beam of neutrons, and the appropriate wavelength must be selected. This is done in several ways. For high-resolution crystallographic studies, a crystalline monochromator is used which defines the incident wavelength with an accuracy of 10^{-2} to 10^{-3}. For solution studies and low-resolution crystallography, a broader wavelength band is acceptable. This is achieved by velocity selectors which select the neutrons mechanically. This is feasible because neutrons of wavelengths 1–10 Å have speeds of several hundred meters per second. Such a selector is described in Ibel (1976). The wavelength band is, according to specifications of the machine, between 4% and 40%. This represents, compared with the crystal monochromator, a gain in intensity by two

to three orders of magnitude and compensates for the weakness of neutron sources.

2.4.2. Experimental Setup

For X-ray crystallography, the setup appropriate to the large size of the viruses is described by Schutt and Winkler (1977). For solution studies, which are essentially measurements at small angles, a good collimation of the beam must be achieved. With neutrons, this is easily achieved with long distances between the neutron source, the sample, and the detector. A camera with axes distances as large as 40 m is described by Ibel (1976). The wavelengths used with neutrons are in the range of 1–20 Å. Because the relevant parameter is the scattering vector, s, which is inversely proportional to the wavelength, the scattering which takes place at 1′ of arc with X-rays ($\lambda = 1.54$) will be at 13′ with 20-Å neutrons. This is quite useful for the study of large viruses. Thus with X-rays one should have an even better collimation. This is usually achieved with a focusing camera which uses a bent monochromator to focus in one direction and a mirror to focus in the other, as first developed by Huxley and Brown (1967).

2.4.3. Samples

Single crystals suitable for X-ray diffraction have linear dimensions of 0.1–1 mm. Similar crystals can be used for low-resolution neutron crystallography. Crystals are mounted in a capillary following methods used in protein crystallography. Data collection with X-ray usually requires a large number of crystals because they are destroyed by the X-ray beam. In a neutron beam, crystals appear stable after years of exposure.

For solution studies, the physical form of the sample is somewhat a function of the quantity of virus which is available. If large quantities can be prepared, a pellet of viruses about 1 mm thick can be used for both X-rays and neutrons. This does not apply to the inner part of the scattering curve (region of the radius of gyration), which must be measured with concentrations of 1–5 mg/ml to avoid interferences between particles. If the virus can be prepared only on a limited scale, concentrations of 10 mg/ml are adequate (although not optimal) for low-particle-weight viruses (small plant viruses); for large viruses

(particle weight of 100 million, for instance), a concentration of 1 mg/ml is quite adequate.

3. STRUCTURES OF SPHERICAL VIRUSES

The largest amount of structural information is available on the icosahedral isometric viruses. This is expected because many of these viruses crystallize easily, and solution scattering studies, as we have seen previously, can provide important structural information on spherical viruses. We shall first discuss tomato bushy stunt virus with which all the methods we have described before have been used.

3.1. Tomato Bushy Stunt Virus

Tomato bushy stunt virus (TBSV) is a small plant RNA virus, built from 180 copies of a polypeptide chain of 40,000 molecular weight, with single-stranded RNA of 1.4×10^6 daltons. This virus has been studied with X-ray small-angle scattering (Harrison, 1969), X-ray diffraction (Harrison and Jack, 1975; Winkler *et al.*, 1977; Harrison *et al.*, 1978), neutron small-angle scattering (Chauvin *et al.*, 1978*b*), and neutron diffraction (Timmins, private communication). A review of the results obtained by these various techniques is quite illuminating; it shows their respective merits and limitations as well as their complementarity.

The X-ray diffraction study was done in successive stages, with increasing resolution. The first two maps of the virus were at resolutions of 28 Å (Jack *et al.*, 1975) and 16 Å (Harrison and Jack, 1975). With that level of resolution, the dimer clustering of the protein subunits previously established by electron microscopy (Finch *et al.*, 1970) is visible. As the electron density of RNA (unhydrated) is much higher (0.59 $e/Å^3$) than the protein electron density (0.44 $e/Å^3$), an attempt was made to localize the RNA as the region of highest density. The conclusion was drawn that the RNA was strongly imbedded within the protein subunits at radii of 100–150 Å. The map at 5.5 Å (Winkler *et al.*, 1977) was already much easier to interpret. It is characterized by an electron density concentrated between radii 110 Å and 170 Å. The protein subunit was found to be made up of two parts. An outer part between 140 and 170 Å is the part of the subunits which clusters in dimers and is made essentially of β sheets. The inner part between 110 and 140 Å shows a very dense packing of subunits, but the boundary of

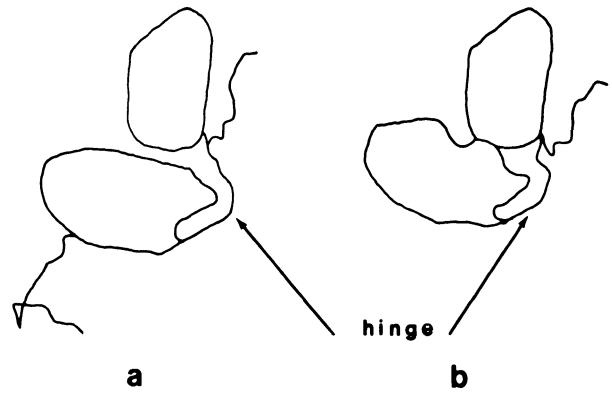


Fig. 9. Organization of the coat protein of TBSV in two domains. This organization is deduced from X-ray crystallography (Harrison *et al.*, 1978). The two domains are connected by a hinge: In TBSV all subunits are associated to form dimers. (a) Organization of the two domains of the proteins forming a dimer across the exact 2-fold axis (icosahedral and crystallographic symmetry), whereas (b) shows the two domains to be in a different arrangement when the dimer has only a local 4-axis. In molecular terms, the traduction of the quasiequivalent positions, as discussed by Casper and Klug (1962), is made possible by this hinge. Part of the protein is missing; which has not been revealed by crystallography (see text).

each subunit is clearly identified. The inner part of the protein is connected by a hinge to the outer part, and this hinge is in a different state for proteins related by strict twofold axis, or simply by a local axis. (In an icosahedron, there are 15 genuine two fold axes, whereas the existence of 90 dimers gives altogether 45 twofold axes, of which 30 are only local axes.) This is shown in Fig. 9. At strict twofold axes, the hinge is open, leaving a cleft which does not exist between subunits connected by a local axis of symmetry. This observation is important as it explains on the level of the protein structure how the principle of quasiequivalence of Caspar and Klug (1962) is used to build a spherical virus. On this 5.5 Å map, density found in the cleft between the subunits was attributed to RNA, so that a major amount of the RNA appeared to be located between 110 and 120 Å, and a role was attributed to it in opening the hinge.

The analysis at 2.9 Å has shown the correctness of the interpretation of the 5.5 Å map in terms of two domain subunits with a hinge, but the attribution of the density in the cleft to RNA turned out to be incorrect. At 2.9 Å, it is possible to follow the polypeptide chain, and the following important conclusions were drawn:

1. No RNA is visible. All the electron density between 110 and 170 Å is given by the protein.

2. At least 50 amino acids are not present in the map.
3. There are 33 amino acids visible in only one out of three subunits. These are the subunits related by a true three fold axis, and the three polypeptide strands of those subunits are strongly interconnected, forming a sort of annulus.

The absence of features in the map (all the RNA and some 15% of the protein) means that these parts of the virus are not organized to form a single crystal (see discussion above), and that they are disordered.

Neutron small-angle scattering (Chauvin *et al.*, 1978*b*) has shed some light on the part of the virus not observable by X-ray diffraction. In that study, the virus was approximated to a spherical object, and no attempt was made to analyze the deviations from sphericity due to icosahedral symmetry because electron microscopy and X-ray diffraction are clearly more powerful for this purpose. Figure 10 shows the small-angle scattering measured with various amounts of D_2O in the solvent. The variation of intensities and peak position are due to the changes with D_2O of the respective contributions of RNA and protein to the scattering. Those curves were analyzed by a model for the virus made up of successive concentric shells, to give the composition of each shell. Four shells were necessary with the following size and composition (in volume):

30–50 Å	0% protein	38% RNA	62% water
50–80 Å	55% protein	2% RNA	43% water
80–110 Å	3% protein	31% RNA	66% water
110–158 Å	78% protein	1% RNA	21% water

Because TBSV is made of only one type of polypeptide chain, those results establish that the protein subunit is made up of two parts. A first part between 110 Å and 158 Å is the one analyzed in detail by X-ray diffraction and found to be made of two domains as shown in Fig. 9. No RNA is found in that shell (1% is not a significant amount). A second part is located between 50 and 80 Å, namely 30 Å away from the first part. The connection between the two domains must be a strand of some ten amino acids in an extended configuration. Most of the RNA (85%) is located in this 30 Å gap between the two protein domains. This is summarized in Fig. 11. Previous experiments with X-ray small-angle scattering (Harrison, 1969) did not reveal this aspect of the structure. This is due to the already discussed inability of X-rays to distinguish between hydrated RNA and protein. This difficulty is true both in X-ray small-angle scattering and in X-ray diffraction. We

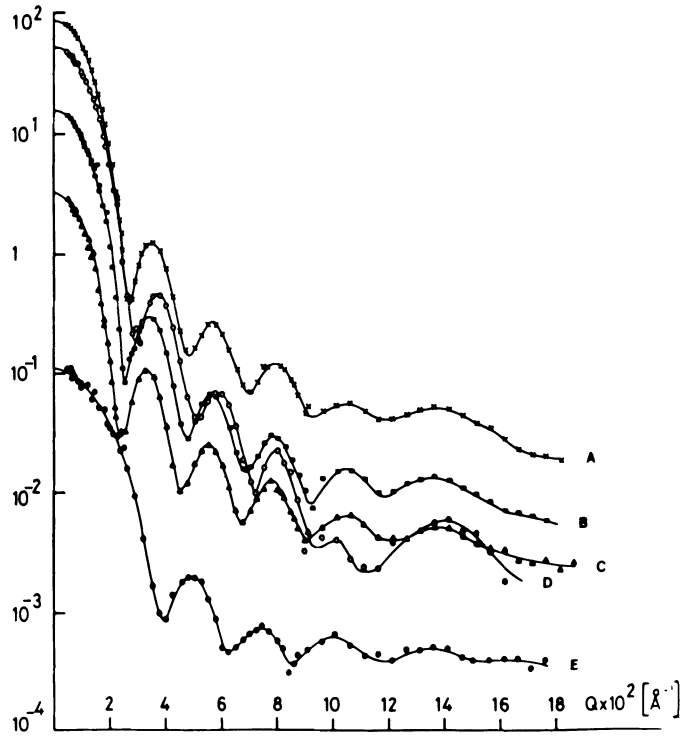


Fig. 10. Neutron-scattering curves from TBSV solutions with various amounts of D_2O in (A) 100%, (B) 68%, (C) 55%, (D) 0%, (E) 41% solution. On the horizontal axis, Q is $2\pi s$.

have seen that, in low-resolution maps and even at 5.5 Å, electron density features were incorrectly attributed to RNA. It is only a high-resolution X-ray map, where the polypeptide chain is clearly visible, which gives the correct information.

To summarize the existing knowledge on TBSV structure, the three-dimensional structure of about 85% of the polypeptide chain is known in a way quite comparable to what is known in normal soluble globular protein. This structure is made up mostly of β-pleated sheet, it is split in two domains with a hinge, and it is used to form the icosahedral shell. The absence of knowledge of the amino acid sequence makes an analysis of the chemical links between subunits difficult. There is no RNA embedded in that protein shell. The remaining 15% of the polypeptide chain, as well as the RNA, is disordered and does not follow icosahedral symmetry. This deviation from icosahedral symmetry appears at 110 Å inward on the 5.5 Å map; no density is visible at smaller radius. Most of the RNA is located in the next 30 Å where it must interact with a completely extended polypeptide chain. This orga-

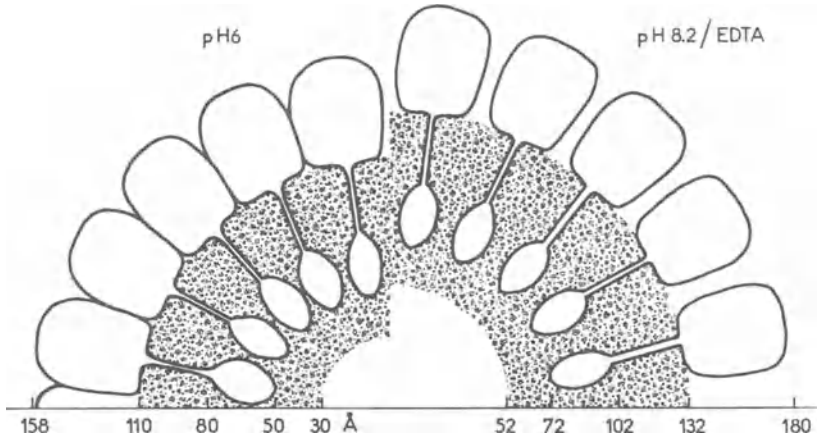


Fig. 11. Internal organization of TBSV. The left part shows the virus at pH 6 (native state). The outer part of the shell (between 110 and 158 Å) is that analyzed in detail by X-ray diffraction and shown in Fig. 9. TBSV protein appears to be made altogether of three domains. The RNA is shown as a shadowed area and is mostly between 80 and 110 Å. The right part of the drawing shows the organization of the virion in a swollen state.

nization makes possible specific interactions between amino acids and bases which would be impossible within strict icosahedral symmetry. Nothing is known of the organization of the RNA within the domain where it is known to be located.

Neutron single-crystal diffraction could give some information. Strong electron density features are present on a low-resolution X-ray map (16 Å) between 80 and 110 Å. Thus neutron scattering density maps obtained with various amounts of D_2O in the crystal will say how the RNA is organized in that shell (Bentley *et al.*, 1978; P. Timmins, private communication).

3.2. Southern Bean Mosaic Virus

Southern bean mosaic virus (SBMV) is a small plant virus with $T = 3$. The protein subunit has a molecular weight of 28,250 and the RNA has a molecular weight of 1.4×10^6 (Tremaine, 1966). The virus has been crystallized (Akimoto *et al.*, 1975) in various forms, and an X-ray diffraction study has been done with those crystals (Johnson *et al.*, 1974, 1976; Rayment *et al.*, 1978; Suck *et al.*, 1978) in successive steps of improved resolution as was done with TBSV. In the electron microscope, the virus appears rather smooth; so far, no analysis has

been made of the type of clustering of the protein subunits. The analysis of the data at 22.5 Å (a resolution comparable to that obtained in electron microscopy) suggests that the protein must be bilobal, with one lobe clustering in hexamers–pentamers (like the clustering in TYMV) and the other lobe (about one-third of the total molecular weight) clustering around the quasi-threefold axis. This organization explains the smoothness of the virion seen in the electron microscope. This organization was confirmed in the 11 Å resolution map (Rayment *et al.*, 1978). At 5 Å resolution (Suck *et al.*, 1978), a more detailed analysis was given, and, in particular, part of the electron density has been analyzed in terms of the phosphate groups of the RNA, with the claim that at least 36% of the RNA has an ordered structure. The situation found with TBSV (see above) shows that one must be careful before taking for granted conclusions drawn from a 5 Å map. Recently, the structure of SBMV has been determined with the same resolution as that of tomato bushy stunt virus (Abad-Zapatero *et al.*, 1980). A strong similarity exists between the structure of those two viruses. Indeed, the folding of the polypeptide chain of the coat protein of SBMV is nearly identical to the folding of the chain in the inner domain of the coat protein of TBSV. In both viruses the RNA, as well as some 50–60 amino acids, is invisible in the electron-density map. The main difference between the two viruses is the protruding domain at the twofold axis which is present in TBSV but not in SBMV. This strong similarity between two viruses is supported by neutron small-angle scattering (Witz, Timmins, Kruse, and Jacrot, unpublished work). Such a similarity between two viruses which have no known relationship is rather unexpected. Argos (private communication) has speculated, on the basis of the known sequences of coat proteins, that the organization of the polypeptide chain found in TBSV and SBMV may be general for plant viruses with strong protein–protein interactions. The work now being done on eggplant virus (Colman, communication at the Seventh Aharon-Katzir-Katchalsky Conference, 1980)—two tymoviruses—will show whether this hypothesis is correct.

3.3. Satellite Tobacco Necrosis Virus

Satellite tobacco necrosis virus (STNV) is a small, icosahedral virus which multiplies only in the presence of tobacco necrosis virus. It is a $T = 1$ particle with 60 identical subunits of molecular weight

21,600 and a single-stranded RNA of 1200 nucleotides (Fiers, personal communication to B. Stranberg, Rees *et al.*, 1970). In a $T = 1$ particle, all proteins can be in strictly equivalent position inside the capsid, a situation which is not possible with a $T = 3$ particle (Caspar and Klug, 1962). Its small size and the knowledge of the sequences of both the nucleic acid and the protein (Ysabaert *et al.*, 1980; Henriksson *et al.*, 1980) make it quite suitable for a detailed structural investigation.

STNV crystallizes easily, giving samples diffracting to high resolution (Fridborg *et al.*, 1965); the structural investigation has been carried through successive stages of resolution (Akervall *et al.*, 1971; Strandberg *et al.*, 1975; Unge *et al.*, 1979). The latest stage is an analysis at 4 Å; at that level, it is not possible to follow the polypeptide chain. However, because the sequence of the protein and the position of the heavy atoms on that sequence used for the crystallographic study are known (Unge and Strandberg, 1979), it is possible to draw the following conclusion:

1. The protein subunit is made up of two domains: the main one between radius 62 and 93 Å looks similar to a globular protein. From that domain, an arm, which is likely to be an α helix, is pointing to the interior of the virion between 62 and 44 Å. Those arms are in contact around the 3-fold axis.
2. No RNA is visible and also no density is found corresponding to the 15 residues on the *N*-terminal side of the polypeptide chain.

This organization, which is schematized in Fig. 12, has obviously strong similarities with that found for TBSV. The viral protein is made of two parts: one part is making the shell. In the case of TBSV, this first part has a hinge to allow for the adaptation of the subunits to the principle of quasi-equivalence (Caspar and Klug, 1962). In the case of STNV, when all subunits are in identical surroundings, this hinge is unnecessary and absent. The second part of the viral protein is that in contact with the RNA. It is organized to maximize the number of contacts between the RNA and the polypeptide chain. The N-terminal part of the peptide chain does not follow icosahedral symmetry, nor does the RNA. How general this organization is remains an open question waiting for more experimental data. Those data should come partly from the X-ray single-crystal work under way (SBMV, see above; cowpea mosaic virus and belladona mottle virus, private communication from Michael Rossmann) and partly from solution studies, mainly with neutrons, which allow a more systematic survey. In the

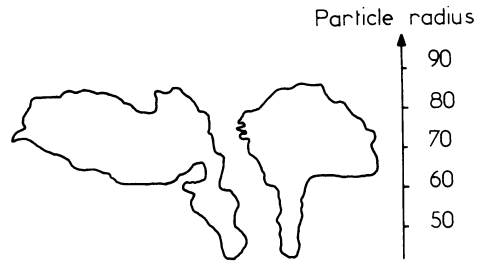


Fig. 12. Schematic drawing of the protein subunit of STNV as seen from two different directions normal to the 3-fold axis. There are two domains but without a hinge. As in TBSV, part of the protein is not revealed by X-ray diffraction. From Unge *et al.* (1979).

case of STNV, such investigations have been done both with X-ray (Sjöberg, 1977) and with neutrons (Chauvin *et al.*, 1977)—showing the main domain of the protein subunit and localizing the RNA—but these studies have not shown the tail of the protein. In the neutron studies, this is probably due to the small amount of experimental data, which is limited by the small concentrations that can be obtained because of the low solubility of that virus.

3.4. Other Small RNA Plant Viruses

Although many small RNA plant viruses can be easily crystallized, the three discussed above (TBSV, SBMV, and STNV) are the only ones on which detailed crystallographic investigations have been reported. On other viruses, the structural information is based only on X-ray or neutron scattering from solutions or, in a few cases, on very low resolution X-ray crystallography.

3.4.1. Turnip Yellow Mosaic Virus

Turnip yellow mosaic virus (TYMV) is a $T = 3$ particle with a subunit of 20,130 daltons, and a piece of RNA of about 1.9×10^6 daltons. In the infected cell, empty capsids (often referred to as natural top components) coexist with complete infective particles. Both empty and complete particles can be crystallized. The unit cell of both crystals is identical, and the diffraction patterns are also very similar except at very low resolution (less than 20 Å). Those two facts strongly suggest (Klug *et al.*, 1966) that the empty capsid is identical to the capsid of the virus. This was later confirmed by neutron scattering from solutions (Jacrot and Witz, unpublished). The scattering by the natural top

component is identical to that given by the virus in a solvent which makes the RNA invisible.

The comparison between the X-ray diffraction patterns given by the virus and the empty capsids has been used by Klug *et al.* (1966) to learn about the RNA and its relation with the capsid. This was done in several ways. One was a model calculation used to fit the data of the top component, then that of the complete virus. To make these calculations possible, the models were point models, namely, the protein and the RNA were replaced by points distributed on spheres with icosahedral symmetry. The other approach was to do diffraction of TYMV crystal in 4 M ammonium sulfate. With this high salt content, the electron density of the solvent is close to that of the protein, and the diffraction pattern is then dominated by the RNA. Those two methods, although not giving exactly the same answer, indicated a very strong penetration of the RNA within the protein shell, and a model was produced showing 32 bumps of RNA penetrating some 20 Å within the protein shell in the middle of the hexamer and pentamer clusters.

At a later date, however, neutron small-angle scattering (Jacrot *et al.*, 1977) showed this model to be incorrect. Using the methods already explained, it was shown that the protein forms a very dense shell which is hardly penetrated by the RNA (about 5 Å). Figure 13 shows the structure of the capsid deduced from electron microscopy and from X-ray diffraction (Klug *et al.*, 1966), with the localization of the RNA deduced from neutron small-angle scattering. The X-ray results also suggest that part of the RNA does have the icosahedral symmetry, a fact that neutron small-angle scattering cannot reveal.

A priori, one would expect that the matching of electron density of the protein in the case of X-ray or that of its scattering density in the

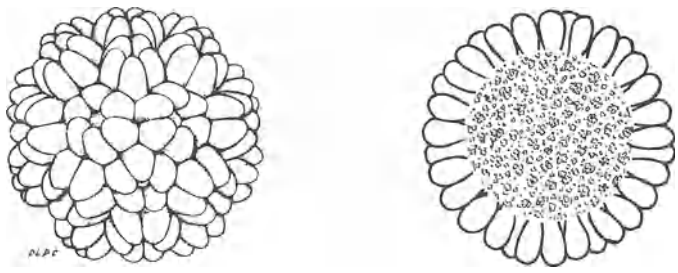


Fig. 13. Structure of turnip yellow mosaic virus. The left part shows the organization of the subunits in hexamers and pentamers established by Klug *et al.* (1966). The right part shows the interior of the virion, where the RNA is shown as a shadowed area with very little interpenetration with the protein subunits.

case of neutrons should give identical results. This is not so, because, when adding D_2O to the solvent, this distribution is done in a uniform way, and the solvent has the same scattering density all over the sample. When adding salt, this salt is likely to be excluded from the immediate vicinity of the protein (hydration shell), so that one has within the sample a nonuniform distribution of the salt, and consequently of the electron density within the solvent. This may strongly perturb the diffraction and make the analysis difficult. A similar situation has been found with TBSV. This again shows that, if X-ray diffraction is unique in high resolution, its interpretation at low resolution is difficult.

3.4.2. Wild Cucumber Mosaic Virus

Wild cucumber mosaic virus (WCMV) is serologically related to TYMV, and was the object of an early investigation by X-ray small-angle scattering (Anderegg *et al.*, 1961). In this very careful work, the scattering from a solution of viruses was compared to that of the top component (natural empty capsid). The radial distributions of electron densities obtained from the two samples (Fig. 14) were interpreted in terms of a protein shell of some 35 Å, with the RNA filling the 210 Å hole left inside the protein shell. This architecture turns out to be very similar to the one found by neutron scattering for TYMV (Jacrot *et al.*, 1977), a result in good agreement with the biological relations between the two viruses.

The data of Anderegg *et al.* (1961) have been analyzed further by Jack and Harrison (1975), who have looked at the deviation of the scattering curve from that given by a sphere. Those deviations are due to the icosahedral organization of the subunit. Jack and Harrison were able to show that those subunits are clustered in hexamers and pentamers like in TYMV.

3.4.3. Bromoviruses

3.4.3a. Bromegrass Mosaic Virus

Bromegrass mosaic virus (BMV) is the prototype of bromoviruses: $T = 3$ particles, with a subunit of 20,300 daltons, and a divided genome. This was the first virus for which a pH dependence of the size was demonstrated (Incardona and Kaesberg, 1964). This pH depend-

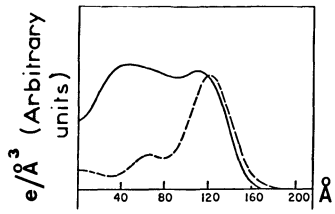


Fig. 14. Radial distribution of electron density in wild cucumber mosaic virus (solid line) and empty capsids of the virus (broken line). From Anderegg *et al.* (1961).

ence of the architecture of the virus has been investigated in some detail with neutron scattering from solution (Jacrot *et al.*, 1976, Jacrot *et al.*, 1977; Chauvin *et al.*, 1978*a*). These studies show that the native form of the virus at pH 5.5 has a diameter of 270 Å, with a limited interpenetration of the RNA in the protein shell (10–15 Å). At this pH, the dimensions of the capsid are independent of ionic strength and divalent ions, whereas the RNA is slightly more compact at higher ionic strength. At basic pH, the virus is swollen, with a diameter between 290 and 310 Å depending on ionic strength and divalent ions. The penetration of the RNA in the capsid in this swollen form is more pronounced.

In all states, the virus has a large central hole (100 Å at low pH, 150 Å in diameter at high pH), which implies a localization of the RNA on a rather narrow shell in contact with penetration in the protein shell. Those data were analyzed with the implicit hypothesis that the proteins are forming a simple shell. If the protein were indeed like those of TBSV, the data should be somewhat reinterpreted.

BMV is easily crystallized, and a detailed X-ray analysis may become available (Rossmann *et al.*, 1973).

3.4.3b. Broad Bean Mottle Virus

Broad bean mottle virus (BBMV) is a $T = 3$ particle with a subunit of about 21,000 daltons and 22% RNA (divided genome). It has been investigated by several groups using X-ray small-angle scattering (Finch *et al.*, 1967; White and Fischbach, 1973; Fischbach and Anderegg, 1976) and low-resolution X-ray diffraction (Finch *et al.*, 1967). The low-resolution data were used to prove the icosahedral symmetry of the virus. The solution work shows that the virus has a diameter between 260 Å and 290 Å, with a central hole of some 100 Å diameter. In very gross terms, this virus is quite similar to BMV, as expected for two viruses of the same family.

3.4.4. Cucumber Mosaic Virus

Cucumber mosaic virus (CMV) is also a $T = 3$ particle with divided genome and a protein subunit of 26,000 daltons. Neutron scattering from solution (Jacrot *et al.*, 1977) shows a diameter of 290 Å. The protein shell is rather thick (65 Å), and the RNA penetrates some 20 Å inside this shell. This architecture, which is very different from that of TYMV (in which the RNA hardly penetrates the protein shell), can be correlated to the fundamental importance of protein–RNA interaction in stabilizing this virus (ribonuclease destabilizes the virus; Francki, 1968), whereas TYMV is stabilized mostly by protein–protein interaction as shown by the isomorphism of the empty capsid to the virus. The comparison between the properties of those two viruses has been discussed in detail by Kaper (1975).

3.5. General Remarks on the Structure of Small RNA Plant Viruses

It is tempting to try to deduce from all the data presented above some rules on the architecture of this class of viruses. This can be done only in a very tentative way, the amount of detailed information being still very limited.

1. Viral proteins seem to be made up of several domains. This allows the assembly in an icosahedral shell according to the principle of quasi-equivalence. The data also suggest that the part of the protein interacting with the RNA is organized in a way which is not that found in a globular protein. Part of the protein seems even to be disordered and to deviate from icosahedral symmetry. This is likely to be useful to enhance protein–RNA interactions, as most, if not all of the RNAs seem to depart also from that symmetry.

2. Near the interior of the virus, the 3-fold (or the quasi-3-fold) axis seems to play an important role, with strong interactions between subunits at that level. This may suggest a morphogenesis of the virion from the assembly of three subunits (or three dimers).

3. Swelling of virions with pH has been reported in several cases (Incardona and Kaesberg, 1964 for BMV; Hsu *et al.*, 1977 for SBMV; Harrison, private communication for TBSV), and may be a rather general phenomenon. The organization described for TBSV provides an easy understanding of that process. Indeed, neutron scattering in that case (Witz and Jacrot, unpublished work) shows that the swelling is a global displacement (some 20 Å) of groups of RNA and proteins of the

low pH structure (see Fig. 11). This unit is likely to be a trimer with the associated RNA. In some conditions, Rayment *et al.* (1979) have obtained crystals of swollen SBMV. The comparison of the details of that structure with the matrix one should be interesting.

The case of TYMV and its family may be a rather special one. All data support the idea that, in that virus, the proteins form a shell which is in contact with the RNA only on its interior surface. This is also the family in which empty capsids are found *in vivo*.

3.6. DNA Plant Viruses

Much less is known about DNA plant viruses than about RNA plant viruses. Cauliflower mosaic virus (CaMV) has been investigated by neutron scattering (Chauvin *et al.*, 1979). The architecture has been found to be specially simple: a protein shell 65 Å thick internally coated with a mono (or possibly double) layer of DNA.

3.7. Spherical Bacteriophages

3.7.1. R17

R17 (and closely related viruses such as fr and MS2) is an icosahedral $T = 3$ particle with a unique type of protein subunit of 13,750 daltons, and single-stranded RNA (1.23×10^6 daltons for MS2). An important difference with small plant viruses is the presence of one copy of the A protein, which is likely to be bound to the RNA. No crystallization of this virus has been reported, and all structural information is based on small-angle scattering of X-rays (Fischbach *et al.*, 1965; Zipper *et al.*, 1971) and neutrons (Jacrot *et al.*, 1977). There is a general overall agreement between these experiments which reveals the following architecture: the protein shell is very tightly packed and is about 30 Å thick. With a virus diameter of 265–270 Å, the RNA, following neutron results, does not interpenetrate the protein shell, or penetrates very slightly following X-ray results (Zipper *et al.*, 1971). The neutron scattering reveals a big hole (120 Å in diameter) in the center of the particle. Thus the virus appears as a very dense protein shell internally coated with the RNA.

3.7.2. P22 and λ Phages

P22 and λ phages are not strictly isometric as they have a tail, but the contribution of this tail to scattering is negligible. They are DNA-containing bacteriophages (26×10^6 for P22; 30×10^6 for λ) with a diameter of 600 Å for P22 and 540 Å for λ. The morphogenesis of these viruses is a many-step process, with intermediate particles which can be isolated from mutants. The virus and some of those intermediate particles have been investigated by X-ray small-angle scattering (Earnshaw *et al.*, 1976; Earnshaw and Harrison, 1977). The large size of these viruses compared with small plant viruses or R17 has two consequences for X-ray scattering: the first one is that deviation from spherical symmetry will be less important, as the irregularities on the surface are separated by distances which are small compared with the diameter of the virus, so that the spherical approximation will be valid up to the tenth subsidiary maximum. This makes the calculation of the radial distribution $f(r)$ rather accurate.

The second practical point is that, with the X-ray camera used by those authors, the central maximum (and possibly the first subsidiary maximum) cannot be recorded (the first zero is at some 10′ of arc). The analysis is therefore done by calculating from a model what this central maximum should be. It is a drawback that would not exist with neutron scattering, as long wavelength neutrons are available which are better matched to those large viruses (see later on frog virus 3). However, data extending well inside the central maximum and taken with a different X-ray camera have been reported by Feigin *et al.* (1974).

It appears that the P22 prohead, made of coat and scaffolding proteins, has a diameter 60 Å smaller than the phage, but if the scaffolding proteins are removed from the prohead its diameter becomes that of the virus. Also, the empty virus (no DNA) obtained from a mutated phage has the same diameter. The particles formed in the absence of scaffolding proteins have also been analyzed (Earnshaw and King, 1978) and were found to have the size of the prohead.

The analysis is based on the scattering pattern up to about 60 Å (scattering vector 0.017). Beyond that point, nonspherical terms start to appear due to the organization of the protein on the shell. At still higher angles, features appear only in the complete phages. The main one is a broad maximum at 24 Å which is likely to be due to DNA packaging. A close examination of this peak shows that it is modulated by a series of regularly spaced ripples. Similar observations were made

with the phage λ. The interpretation of Earnshaw and Harrison (1977) is that the 24 Å maximum arises from the distance between DNA strands packaged in the phage. Indeed, with mutants of λ which have more or less DNA, this peak is shifted to a larger angle (26 Å) or a smaller angle (23 Å). The modulations are attributed to correlation between DNA strands on appropriate sides of the phage. A similar peak at 24 Å has been reported for the nonspherical phage $\phi 29$ (Subirana *et al.*, 1979). In this latter case, the X-ray scattering has also been used to determine the size of the virus. This can be done even if the virus is nonspherical by fitting a model to the data. This procedure is very reliable as the scattering pattern still has well-defined maxima and minima. The sizes of viruses determined in that way have been shown by Earnshaw *et al.* (1978) to be usually larger than those obtained from electron microscopy. It must be stressed that diffraction and scattering techniques are, for this purpose, free of artifact, and sizes obtained by X-rays or neutrons must be preferred to those deduced from various electron microscope techniques; in this latter case, distortions due to staining, drying, and flattening are expected.

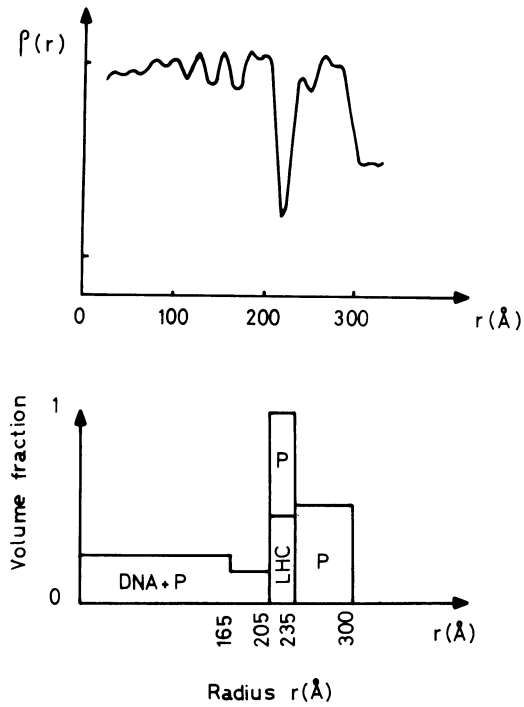


Fig. 15. Structure of PM2. The top part is the radial distribution measured by X-ray small-angle scattering. From Harrison *et al.* (1971*a*). The bottom part is the distribution of protein (P), DNA, and lipid (LHC) expressed as a volume fraction. From Schneider *et al.* (1978).

3.7.3. PM2

The PM2 bacteriophage contains lipids. In X-ray scattering (Harrison *et al.*, 1971*a*), it appears as a particle in which spherical symmetry is a very good approximation, and a very good radial distribution of electron density was derived. This distribution is shown in Fig. 15; its main feature is a narrow peak of very low density at 220 Å which is attributed to the lipids, which have a much lower electron density than protein and DNA. The width of this peak suggests that these lipids are organized in a bilayer. The model was later refined with neutron scattering (Schneider *et al.*, 1978). As already explained for tomato bushy stunt virus, the use of data collected with various amounts of D_2O in the solvent makes possible a chemical analysis of shells in a spherical organization. In this way, it was possible to show that the lipid bilayer is penetrated by proteins which occupy nearly 50% of its volume, and that these proteins are coming from the central core (made of DNA and protein) and not from the outer shell.

3.8. Spherical Animal Viruses

Very little structural work with X-rays or neutrons has been done with animal viruses because of the small quantities of purified viruses usually available and the lack of facilities for virus containment in most biophysical laboratories. These difficulties will have to be overcome because diffraction techniques are the only appropriate way to get reliable information on the internal organization of a virus and on the size of the virion.

Several animal viruses have been crystallized: poliomyelitis virus, Coxsackie virus, wart virus, polyoma virus etc. Some of these crystals have been used to prove the validity of the theory of icosahedral symmetry. The X-ray diffraction by poliomyelitis virus was used by Finch and Klug (1959) to show that the building principles of this animal virus were the same as those of the plant viruses previously investigated by the same authors, and that there are no fundamental structural differences between small spherical plant viruses and animal viruses.

Some other viruses have not yet been crystallized *in vitro* but are found to form crystalline lattices in infected cells. For instance, this is true of adenoviruses (Henry *et al.*, 1971). Components of adenoviruses (hexons, fibers, etc.) have also been crystallized (Franklin *et al.*, 1971), and these crystals have been analyzed by standard protein crystal-

lographic techniques (Burnett *et al.*, 1978). One may hope that combination of the knowledge of the structures of those pieces with other information obtained by electron microscopy and biochemistry will eventually provide the structure of the adenoviruses. A similar approach is used for influenza virus. The structure of the protruding part of the hemagglutinin had been solved by high resolution X-ray crystallography providing the localization of the antigenic sets (Wiley, personal communication). So far, the only animal viruses which have been investigated by X-rays or neutrons (apart from the already mentioned pioneer work on poliomyelitis virus) are polyoma virus, Sindbis virus, the closely related alphavirus Semliki forest virus, and frog virus 3, a cytoplasmic DNA virus. We shall review the information obtained on these three virus families.

3.8.1. Polyoma Virus

The general principles of the structure of polyoma virus have been described in a previous volume of this series (Finch and Crawford, volume 5). This virus, with a diameter of 490 Å, has a capsid with a triangulation number T = 7 which has been carefully studied by electron microscopy. Empty capsids are found associated with the virus, and both the virus and those empty capsids can be crystallized. A preliminary X-ray diffraction study has been reported recently (Adolph *et al.*, 1979). The data show that the virus particles and the empty capsids crystallize isomorphously; consequently, a comparison between the electron density maps given by both crystals will eventually reveal the structure of the core, which is made of DNA associated with histones.

3.8.2. Sindbis Virus and Semliki Forest Virus

Sindbis and Semliki Forest viruses are closely related alphaviruses which are characterized by a nucleoprotein core that becomes coated by a lipoprotein envelope when budding through the surface of the cell. Semliki Forest virus has been crystallized, and a high-resolution X-ray diffraction analysis has been considered (K. Simmons, private communication). So far, only solution studies have been done on Sindbis virus by X-rays (Harrison *et al.*, 1971*b*) and on Semliki with both X-rays (Söderlund, private communication) and neutrons (Söderlund, Cuillel, and Jacrot, unpublished work). The X-ray work on Sindbis

virus shows a sharp minimum at 220 Å in the radial distribution of the electron density. This minimum is attributed to a lipid bilayer. The basic organization of the virus is thus a core with RNA and protein surrounded by a lipid bilayer, which is itself surrounded by a protein shell. There is biochemical evidence that the outer protein and the core proteins are in contact through the lipid bilayer, a point not revealed by X-ray small-angle scattering. The reason is the difficulty of putting X-ray data on an absolute scale. The neutron work is now being analyzed and should give quantitative estimates of the amount of proteins in the lipid layer of Semliki Forest virus, which the X-ray work has shown to be nearly identical to Sindbis.

3.8.3. Frog Virus 3

Frog virus 3 (FV3) is a lipid-containing deoxyribovirus. It has been investigated by neutron scattering (Cuillel *et al.*, 1979), a technique which appears to be suitable for very large viruses. FV3 is found to have a diameter of 1580 Å, which is larger than suggested by electron microscopy. The lipids are found in a 40-Å-thick layer at 85 Å inside the virion, leaving a core with a radius of some 685 Å which contains the DNA (100×10^6 daltons) and only a small fraction of the proteins (60×10^6 daltons). Thus this core is highly hydrated, as 80% of its volume is occupied by water. This neutron study has led to a redetermination of the particle weight of the virion (confirmed by other techniques).

3.8.4. Conclusions

There is a wide contrast between the detailed knowledge of several plant viruses and the very poor knowledge of the structure of animal viruses. There are many animal viruses which are not suitable for diffraction study because their shape is not well defined (paramyxovirus, herpesvirus, pox virus) or at least appears poorly defined with the electron microscope. Systematic investigations with solution scattering, mainly with neutrons, could reveal unexpected structural features. Many of the big animal viruses are highly hydrated. They are considerably deformed by shrinkage and flattening during preparation for observation by electron microscopy (Serwer, 1977; Earnshaw *et al.*, 1978). Thus, in our view, sizes of viruses quoted from electron microscopy may be incorrect by as much as 30%. This has strong

implications concerning our understanding of the organization of the virions. Useful information can be obtained with X-ray and neutron scattering on large viruses (e.g., the case of frog virus 3), with quantities of only a few milligrams.

4. HELICOIDAL VIRUSES

Viruses with helical symmetry are found in three classes of viruses:

1. The filamentous bacterial viruses (reviewed by Marvin and Hohn, 1969), which have single-stranded DNA as nucleic acid.
2. The elongated, rod-shaped, or filamentous plant viruses, with single-stranded RNA; they have been classified recently (Veerisetty, 1978).
3. The nucleoprotein core (or nucleocapsid) of paramyxoviruses (and possibly of myxoviruses) which has strong similarities with the helical plant viruses (Finch and Gibbs, 1970). This last group has not yet been studied by X-ray diffraction.

We have seen that the maximum amount of information on these viruses is obtained from oriented gels. It turns out, as already shown by Bernal and Fankuchen (1941), that the elongated shape of those viruses is favorable for the formation of such gels. For tobacco mosaic virus (TMV), which is a long but rigid rod, the parallelism of the virions in the gel is nearly perfect. For filamentous bacteriophages or plant virus (such as narcissus mosaic virus), which are flexible, the parellelism is not as good. Torbet and Maret (1979) have shown that the application of a magnetic field, at least in the case of bacteriophages, can improve the degree of orientation.

To understand well a structure based on helicoidal symmetry, one must first think of a string folded like a helix. This helix is defined by two parameters: the pitch of the helix, and its diameter. Such a simple helix will give a diffraction pattern with maxima and minima on layer lines. These layer lines are perpendicular to the axis of the helix, and their distance is inversely proportional to the pitch of the helix (see Fig. 7a). On the central line (the one on the level of the X-ray beam), the first maximum will be in the center. On successive lines, they will be more and more off the center of the plate (meridional line), forming the characteristic X predicted by Cochran *et al.* (1952) and used to establish the helical structure of DNA. Now let us imagine that we put beads on that string (Fig. 7d). There is no reason why there should be an integral number of beads on one turn of the helix. But eventually,

after several turns (two in the figure), the beads will be in register with those in the first turn. This new helix will have two new parameters: the number of turns, N, necessary to be in register, and the number of beads (or protein subunits) in those N turns. Those two parameters (which are integral numbers) can be reduced to one, the number of subunits per turn, which is not generally an integral number (for TMV it is 16⅓). The X-ray diffraction pattern of this more realistic helix will still have the layer lines given by the pitch of the string, but, in between also (N − 1), layer lines separated by the inverse of the distance between the beads which are in register.

From that discussion, it follows that it will be relatively easy to find the pitch of the helix (the layer lines given by that pitch are stronger than the intermediate line), and the number N defined above. But it is not easy at all to get the number of subunits per turn, although this number is essential, being by itself an important piece of structural information and necessary for the detailed structural analysis mentioned in the introduction. The difficulty of assessing this number of subunits is illustrated by the case of TMV, where numbers of 31 (Watson, 1954) and 37 (Franklin, 1955) were deduced from the data before there was definitive proof that 49 was the correct number (Franklin and Holmes, 1958). Indeed, a knowledge of the length of the particle (deduced from electron microscopy), of its total particle weight, and of the molecular weights of its subunits and of the nucleic acid will provide a good approximation of the number of subunits per turn (as the X-ray easily gives the pitch of the helix) (Klug and Caspar, 1960; Wiseman and Day, 1977; Berkowitz and Day, 1978).

4.1. Tobacco Mosaic Virus

TMV has been the prototype of helical viruses, and the investigation of its structure by X-ray diffraction, initiated by Bernal and Fankuchen (1941), has culminated in a structure deduced from a 4 Å map (Stubbs *et al.*, 1977). The successive steps in solving that structure have been the following:

1. The existence of a periodic structure in the virus deduced from a well-defined X-ray diffraction pattern from oriented gels (Bernal and Frankuchen).
2. The helical arrangement of the subunits, with a pitch of 23 Å and an axial periodicity of 69 Å, deduced from an analysis of the X-ray pattern (Watson, 1954).

3. The localization of the RNA at a radius of 40 Å (Franklin, 1956). This important information was obtained by comparison between the X-ray diffraction pattern of the virus and the polymerized protein which, in appropriate conditions, forms a helix identical to that of the virus. In both patterns, the intensities along the zero layer line (equatorial) can be analyzed to give the average radial distribution perpendicular to the axis of the helix. (This is mathematically quite similar to the analysis of the intensities given by a spherical virus to get the radial distribution as explained above.) The radial distribution of the virus gives a sharp maximum at 40 Å which is absent from the otherwise identical distribution given by the polymerized protein.
4. The determination of the number of subunits in the axial repeat as 49 (Franklin and Holmes, 1958), a point already discussed above.
5. The determination of the electron density map at 6.7 Å resolution (Holmes *et al.*, 1975), and then at 4 Å (Stubbs *et al.*, 1977). This was done using methods derived from the standard crystallography of protein with phasing done with heavy atom derivatives. The method was briefly outlined above.

We have given those successive steps as they show the procedures necessary to solve the structure of an helicoidal virus. In other helical plant viruses, similar steps have been followed but were never pushed to the same level of sophistication (see below). For TMV, it is quite fortunate that the protein polymerizes in a controlled way (Durham, 1972). This was already useful to localize the RNA. It is also useful to get structural information on the subunits beyond the 4 Å resolution, which is likely to be the limit of structural analysis from oriented gels. The protein can be polymerized in a two-layer disk, each layer containing 17 subunits, and these disks can be crystallized and analyzed by X-ray diffraction. This has been done up to a final resolution of 2.8 Å (Champness *et al.*, 1976; Bloomer *et al.*, 1978) using a combination of standard protein crystallographic methods (isomorphous replacement) and the redundancy of information given by the noncrystallographic 17-fold axis of symmetry (Bricogne, 1976). The information given by the virus 4 Å map and the 2.8 Å protein map provide the best knowledge so far obtained for the structure of a virus.

The virus map gives the overall organization of the polypeptide chain, as shown in Fig. 16. The interpretation of the map (as well as that given by the protein disks) is made easier by the knowledge of the

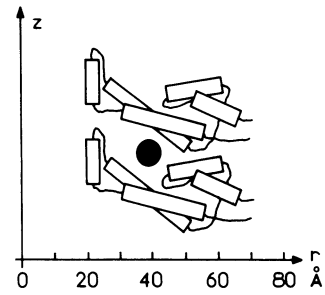


Fig. 16. Schematic drawing of the inner part of TMV. From Stubbs *et al.* (1977). Two subunits are shown one above the other, with the RNA backbone (dark spot) in between. The cylinders represent the parts of the subunits which have an α-helical structure. In reality, the two subunits are not just one above the other, but are tilted to form a helix.

sequence, and, although there are some differences in the interpretation of the two maps, the general features are similar. The polypeptide chain is folded in five α-helices. Four of them are roughly radial, while the fifth one, lying in the center of the virion coat (at about 25 Å radius) is parallel to the axis of the virus. An important feature is that this helix and the connecting strand (altogether 25 amino acids) are invisible on the protein map, indicating that, in the absence of RNA, this part of the chain is disordered. The disk is known to be necessary in the *in vitro* reconstitution of the virus from its constituents, and the reconstitution is known to take place from the inside of the disk. This disorder of the inner part of the protein, which indicates a flexibility of the chain at small radii, is a feature which is likely to facilitate the access of the RNA to its site. This site is at an average radius of 40 Å, as predicted (see above), but the RNA is in fact in a zigzag configuration, the three successive phosphates (the feature dominating in the electron density map) being at 37, 39.5, and 42 Å. The details of the chemical links between the RNA and the protein are still unknown. A virus map could say something, but 4 Å is not a good enough resolution to build an atomic model. The map suggests that there are salt links between the phosphates and arginine residues.

The map obtained with the disk provides information on the nature of the interaction between protein subunits. These interactions involve both hydrophobic contacts and salt links. In the transition from disks to helix, the packing of the subunits is modified, and Bloomer *et al.* (1978) have made suggestions on how this rearrangement takes place.

Recent progress has also been made by combining the information obtained at limited resolution with a virus gel with those from the single crystals of protein disks. Holmes (1980) has proposed a model for the RNA binding site. In that model the binding of the RNA to the protein is described in detail. It involves stereospecific interaction between aspartic acids and ribose groups, hydrophobic interactions with the three bases wrapped around an alpha helix of the protein, specific

hydrogen bonding, and salt bridges. The transition from the protein disk to the viral helix, which is one of the major events in the morphogenesis of the virus, is somewhat better understood through a crystallographic study of single crystals of protein disks with trinucleoside AAG soaked into them (Graham and Butler, 1979). An upward displacement by 3 Å of part of the protein at small radius is well in agreement with a morphogenesis in which the jaw of the protein is closed when the RNA takes its place.

4.2. Other Helical Plant Viruses

No other helical plant virus has been investigated in comparable details, and studies have been essentially limited to the determination of the two helical parameters easily deduced from the X-ray pattern: the pitch of the helix, and the number of turns which have an integral number of subunits.

4.2.1. Narcissus Mosaic Virus and Potato Virus X

Narcissus mosaic virus and potato virus X are filamentous, very flexible viruses about 5500 Å long with a diameter of 130 Å. Early work is from Bernal and Fankuchen (1941), followed by Wilson *et al.* (1973), Wilson and Tollin (1969), and Tollin *et al.* (1968). In both viruses, the helical parameters vary with the amount of water in the specimen. For instance, potato virus X has a pitch between 33 and 36 Å depending on water content. There is in this virus an integral number of subunits in a turn estimated as 10 from arguments involving molecular weight. Narcissus mosaic virus also has a pitch between 33 and 36 Å, but six turns are needed to get an integral number of subunits estimated as 34, also on the basis of molecular weights.

4.2.2. Tobacco Rattle Virus and Barley Stripe Mosaic Virus

Tobacco rattle virus and barley stripe mosaic virus are rigid viruses with diameters of 200–250 Å. Because they are multicomponent viruses, they have lengths that depend on the length of the encapsidated RNA. Preliminary X-ray work (Finch, 1965) has shown that those two viruses have pitches of 25 and 26 Å, respectively, with three and five turns, respectively, to give an integral number of subunits.

With these four filamentous and bacilli-form viruses, the localization of the RNA could not be established on solid ground but was esti-

mated to be at radii around 39 Å for the two filamentous viruses and 60 Å for barley stripe mosaic viruses. Those radii correspond to disposition of the RNA with a phosphorous–phosphorous distance of 5.1 Å. Veerisetty (1978) has shown that the hypothesis that this distance is nearly the same in all helical plant viruses leads to an integral number of nucleotides per subunit, as expected, to fulfill the requirement that each of the protein subunits is in an identical environment. Clearly, more X-ray work on this family of virus would be useful. Neutron scattering should not be too useful with those viruses, which have a limited amount of RNA (5%), except possibly for the determination of mass per unit length.

4.3. Filamentous Bacteriophages

Two filamentous bacteriophages have been extensively studied by X-ray diffraction: fd and Pf1. Both have a diameter of about 60 Å, but fd is 9000 Å long with a single-stranded DNA of 6400 nucleotides, whereas Pf1 is 20,000 Å long with a DNA of 7400 nucleotides. Oriented specimens can be prepared with both viruses. Samples with very good orientation can be prepared by applying a high magnetic field for 30 min on a virus solution (Torbet and Maret, 1979).

Although both viruses have been studied by X-ray, more has been done on Pf1 (Marvin *et al.*, 1974; Marvin and Wachtel, 1975, 1976; Makowski and Caspar, 1978; Makowski *et al.*, 1980). This work is illustrative of the usefulness of combining X-ray diffraction with other physical and chemical methods in solving a structure. The coat protein of Pf1 has 46 amino acids and is known on spectroscopic grounds to be nearly a pure α-helix. The virus itself gives a diffraction pattern characteristic of helical structure with a pitch of 15 Å. The axial repeat is made of five turns (75 Å). We have seen in TMV that those are the two parameters easy to determine, but that the number of subunits in that repeat is extremely difficult to reduce purely from the analysis of the X-ray diffraction pattern.

The X-ray diffraction pattern suggests 22 subunits in those five turns, but Wiseman and Day (1977), on the basis of particle length, DNA content, and DNA length, showed that the number of subunits should be 27 or 28. Makowski and Caspar (1978), on the basis of packing volumes of the virus at different relative humidity, ruled out 28. They also showed that the interlocking of the virions with 27 subunits (but not with 22 or 28) explains observed interference effects. Thus they conclude that there are 27 subunits in five helical turns. This discussion

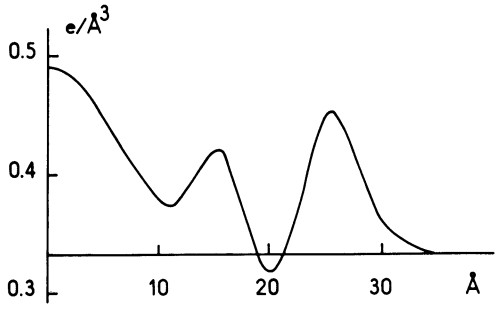


Fig. 17. The radial distribution of the electron density in Pf1. From Makowski and Caspar (1978). The central peak is due to DNA. The two outer peaks are given by the protein and suggest a two-layer organization. This is confirmed by more elaborate fiber diffraction analysis (Makowski *et al.*, 1980).

is not academic at all, because the next stage in solving the structure of the virion is to use the fact that the subunit is nearly a pure α-helix, to build a model, and to find which model will fit the X-ray diffraction. In this procedure, the subunit is approximated to a tube (or a small number of tubes) of 10 Å diameter; clearly, the number of proteins which must be fitted in a given length of the virion is quite critical. A first model was produced (Marvin and Wachtel, 1975) on the basis of the 22 subunits; this model was then revised (Makowski *et al.*, 1979) to allow the packing of 27 subunits. (The exact procedure is to use the model as a constraint for the phases necessary to calculate the electron density. The model thus replaces heavy atom derivatives.) As for TMV, one can calculate from the intensities on the equatorial line the radial distribution of electron density. This is shown in Fig. 17. There are three peaks: the central peak is attributed to DNA; the two peaks at 15 and 25 Å correspond to two layers of α-helices. The final model of the virus is a central core with two strands of DNA surrounded by two shells of α-helices, the first one in close contact with the DNA with an axis nearly parallel to the virion axis. In the second outer shell, the helices are tilted by about 25° relative to this axis. The structure of the DNA and the connectivity between the two helical fragments is not clear.

5. CONCLUSIONS

This chapter shows that knowledge of virus structure has increased considerably during the last 10 years, largely from X-ray diffraction studies and partly from solution studies, mostly with neutrons. Those two approaches are complementary: X-ray diffraction brings a detailed and accurate understanding of the protein–protein interaction and of the architecture of the capsid. Neutron scattering brings crude informa-

tion on the architecture of the interior of the virus, where X-ray diffraction seems to cease being useful.

More work is clearly needed. The general laws of the architecture of the capsid have been derived a long time ago by Caspar and Klug. It is not yet known if there are general principles applicable to the architecture of the interior of the virion, at least for a family of viruses like the RNA plant viruses. The steps in the building of a small, spherical virus are still completely unknown, as well as the process of uncoating of these viruses. It is likely that a knowledge of the structure of the interior of viruses, in particular at the level where the protein capsid is in interaction with the RNA, will be necessary to understand morphogenesis and uncoating. Scattering techniques may also contribute to this understanding through experiments where the kinetics of virus (or empty capsid) assembly is followed. With high-flux neutron sources, useful structural information can be obtained in 200 msec (Cuillel and Jacrot, unpublished work). With the very intense X-ray source provided by synchrotron radiation, there is a real hope to get structural information in 10 msec. This should be important to identify possible intermediates in the building of a spherical capsid.

The structure revealed by diffraction and scattering is a static structure. In reality, it is quite possible that the virus structure is not static and fluctuates around a mean configuration: Caspar (private communication) suggests that the X-ray small-angle scattering deviates too strongly from that given by a sphere and that this is due to this dynamic aspect of the structure. Neutron inelastic scattering is a method in which not only the spatial distribution of scattered neutrons is measured, but also their energy. A dynamic virus structure will manifest itself by an energy change of the neutrons, which may be measurable. Also, viruses may very well have several structures depending on external conditions. This is already well documented for several plant viruses, where a pH-dependent swelling has been observed, and also for several animal viruses, namely, Sindbis virus, Semliki Forest virus, and poliovirus, which have a pH-dependent size. This should be explored in a more systematic way; to do this, small-angle scattering (with X-ray or neutrons) is one of the most efficient approaches. This may also lead to some understanding of the uncoating process.

ACKNOWLEDGMENTS

The author thanks Drs. Caspar, Rossmann, and Strandberg for communication of unpublished results. Dr. Martine Cuillel has been most helpful in the preparation of the drawings.

6. REFERENCES

Abad-Zapatero, C., Abdel-Meguid, S. S., Johnson, J. E., Leslie, A. G. W., Rayment, I., Rossmann, M. G., Suck, D., and Tsukihara, T., 1980, southern bean mosaic virus at 2.8 Å resolution, *Nature (London)* **286**:33–39.

Adolph, K. W., Caspar, D. L. D., Hollingshead, C. J., Lattman, E. E., and Phillips, W. C., 1979, Polioma virion and capsid crystal structures, *Science* **203**:117.

Akervall, K., Strandberg, B., Rossmann, M. G., Bengtsson, U., Fridborg, K., Johannisen, M., Kannan, K. K., Lovgren, S., Petef, G., Oberg, B., Eaker, D., Hjerten, S., Ryden, L., and Moring, I., 1971, X-ray diffraction studies of the structure of satellite tobacco necrosis virus, *Cold Spring Harbor Symp. Quant. Biol.* **36**:469.

Akimoto, T., Wagner, M. A., Johnson, J. E., and Rossmann, M. G., 1975, The packing of southern beam mosaic virus in various crystal cells, *J. Ultrastruct. Res.* **53**:306.

Anderegg, J. W., Geil, P. H., Beeman, W. W., and Kaesberg, P., 1961, An X-ray scattering investigation of wild cucumber mosaic virus and a related protein, *Biophys. J.* **1**:657.

Bacon, G. E., 1975, *Neutron Diffraction*, 3rd ed., Oxford University Press, Oxford.

Bentley, G., Lewit, A., and Timmins, P. A., 1978, A low resolution study of tomato bushy stunt virus by neutron diffraction, *Acta Crystallogr. Sect. A* **34**:S51.

Bernal, J. D., and Fankuchen, 1941, X-ray and crystallography studies of plant virus preparations, *J. Gen. Physiol.* **25**:111.

Bloomer, A. C., Champness, J. N., Bricogne, G., Staden, R., and Klug, A., 1978, Protein disk of tobacco mosaic virus at 2.8 Å resolution showing the interactions within and between subunits, *Nature (London)* **276**:362.

Blundell, T. L., and Johnson, L. N., 1976, *Protein Crystallography*, Academic Press, New York.

Bricogne, G., 1976, Methods and programs for direct space exploitation of geometrical redundancies, *Acta Crystallogr. Sect. A*. **32**:832.

Burnett, R. M., Grutter, M. G., Marcovic, Z., and White, J. L., 1978, Determination of the molecular structure of a viral coat protein: Adenovirus type 2 hexon, *Acta Crystallogr. Sect. A* **34**:S51.

Berkowitz, S. A., and Day, L. A., 1978, Mass, length, composition, and structure of the filamentous bacterial virus, *J. Mol. Biol.* **102**:531.

Caspar, D. L. D., and Klug, A., 1962, Physical principles in the construction of regular viruses, *Cold Spring Harbor Symp. Quant. Biol.* **27**:1.

Champness, J. N., Bloomer, A. C., Bricogne, G., Butler, P. J. G., and Klug, A., 1976, The structure of the protein disk of tobacco mosaic virus to 5 Å resolution, *Nature (London)* **259**:20.

Chauvin, C., Jacrot, B., and Witz, J., 1977, The structure and molecular weight of satellite tobacco necrosis virus: A neutron small angle scattering study, *Virology* **83**:247.

Chauvin, C., Pfeiffer, P., Witz, J., and Jacrot, B., 1978*a*, Structural polymorphism of brome grass mosaic virus: A neutron small angle scattering investigation, *Virology* **88**:138.

Chauvin, C., Witz, J., and Jacrot, B., 1978*b*, Structure of tomato bushy stunt virus: A model for protein–RNA interaction, *J. Mol. Biol.* **124**:641.

Chauvin, C., Jacrot, B., Lebeurier, G., and Hirth, L., 1979, The structure of cauliflower mosaic virus: A neutron diffraction study, *Virology* **96**:640.

Cochran, W., Crick, F. H. C., and Vand, V., 1952, The structure of synthetic polypeptides. I. The transform of atoms on a helix, *Acta Crystallogr.* **5**:581.

Crowther, R. A., and Amos, L. A., 1971, Three-dimensional image reconstructions of some small spherical viruses, *Cold Spring Harbor Symp. Quant. Biol.* **36**:484.

Cuillel, M., Tripier, F., Braunwald, J., and Jacrot, B., 1979, A low resolution structure of frog virus 3, *Virology* **99**:277.

Durham, A. C., 1972, Structure and roles of the polymorphic forms of tobacco mosaic virus protein. I. Sedimentation studies, *J. Mol. Biol.* **67**:289.

Earnshaw, W. C., and Harrison, S. C., 1977, DNA arrangement in isometric phage heads, *Nature* (*London*) **268**:598.

Earnshaw, W. C., and King, J., 1978, A structure of phage P22 coat protein aggregates formed in the absence of the scaffolding protein, *J. Mol. Biol.* **126**:721.

Earnshaw, W., Casjens, S., and Harrison, S. C., 1976, Assembly of the head of bacteriophage P22: X-ray diffraction from head, proheads and related structure, *J. Mol. Biol.* **104**:387.

Earnshaw, W. C., King, J., and Eiserling, F. A., 1978, The size of the bacteriophage T4 head in solution with comments about the dimension of virus particles as visualised by electron microscopy, *J. Mol. Biol.* **122**:247.

Feigin, L. A., Dembo, A. T., and Boyarintseva, A. K., 1974, X-ray small angle studies of dimensions and weight parameters of T2, S_D and DD2 bacteriophages, *J. Appl. Crystallogr.* **7**:164.

Finch, J., 1965, Preliminary X-ray diffraction studies on tobacco rattle and barley stripe mosaic viruses, *J. Mol. Biol.* **12**:612.

Finch, J. T., 1972, The study of virus structures by X-ray diffraction, *Contemp. Phys.* **13**:1.

Finch, J. T., and Gibbs, A. J., 1970, Observation on the structure of the nucleocapsids of some paramyxoviruses, *J. Gen. Virol.* **6**:141.

Finch, J. T., and Holmes, K. C., 1967, Structural studies of viruses, in: *Methods of Virology*, Vol. 3 (K. Maramorosh and H. Koprowsi, eds.), pp. 351–474, Academic Press, New York.

Finch, J., and Klug, A., 1959, Structure of poliomyelitis virus, *Nature* (*London*) **183**:1709.

Finch, J., Leberman, R., and Bergen, J. E., 1967, Structure of broad bean mottle virus. II. X-ray diffraction studies, *J. Mol. Biol.* **27**:17.

Finch, J. T., Klug, A., and Leberman, R., 1970, The structures of turnip crinkle and tomato bushy stunt viruses. II. The surface structure: Dimer clustering patterns, *J. Mol. Biol.* **50**:215.

Fischbach, F. A., and Anderegg, J. W., 1976, Comparisons of the low resolution structure of several small RNA-containing virus, *Biochim. Biophys. Acta* **432**:404.

Fischbach, F. A., Harrison, P. M., and Anderegg, J. W., 1965, An X-ray scattering study of bacterial virus R17, *J. Mol. Biol.* **13**:638.

Fowlks, E., and Young, R. T., 1970, Detection of heterogeneity in plant viral RNA by polyacrylamide gel electrophoresis, *Virology* **42**:548.

Francki, R. I. B., 1968, Inactivation of cucumber mosaic viruses (Q strain), nucleoprotein by pancreatic ribonuclease, *Virology* **34**:694.

Franklin, R. E., 1955, Structure of tobacco mosaic virus, *Nature* (*London*) **175**:379.

Franklin, R. E., 1956, Location of the ribonucleic acid in the tobacco mosaic virus particle, *Nature* (*London*) **177**:928.

Franklin, R. E., and Holmes, K. C., 1958, Tobacco mosaic virus: Application of the

method of isomorphous replacement to the determination of the helical parameters and radial density distribution, *Acta Crystallogr.* **11**:213.

Franklin, R. M., Petterson, V., Averkall, K., Strandberg, B., and Philipson, L., 1971, Structural proteins of adenovirus. V. Size and structure of adenovirus type 2 hexon, *J. Mol. Biol.* **57**:383.

Fridborg, K., Hjerten, S., Höglund, S., Liljas, A., Lundberg, B. K. S., Oxelfelt, P., Philipson, L., and Strandberg, B., 1965, Purification, electron microscopy and X-ray diffraction studies of the satellite tobacco necrosis virus, *Proc. Natl. Acad. Sci. USA* **54**:513.

Graham, J., and Butler, P. J. G., 1979, Binding of oligonucleotides to the disk of tobacco-mosaic virus protein, *Eur. J. Biochem.* **93**:333.

Green, D. W., Ingram, V. M., and Perutz, M. F., 1954, The structure of haemoglobin. IV. Sign determination by the isomorphous replacement method, *Proc. R. Soc. London Ser. A* **225**:287.

Guinier, A., 1963, *X-Ray Diffraction*, Freeman, San Francisco.

Guinier, A., and Fournet, G., 1955, *Small Angle Scattering of X-rays*, Wiley, New York, Chapman and Hall, London.

Harrison, S. C., 1969, Structure of tomato bushy stunt virus. I. The spherically averaged electron density, *J. Mol. Biol.* **42**:457.

Harrison, S. C., and Jack, A., 1975, Structure of tomato bushy stunt virus. III. Three-dimensional X-ray diffraction analysis at 16 Å resolution, *J. Mol. Biol.* **97**:173.

Harrison, S. C., Caspar, D. L. D., Camerini-Otero, R. D., and Franklin, R. M., 1971*a*, Lipid and protein arrangement in bacteriophage PM2, *Nature (London) New Biol.* **229**:197.

Harrison, S. C., David, A., Jumblatt, J., and Darnell, J. E., 1971*b*, Lipid and protein organisation in Sindbis virus, *J. Mol. Biol.* **60**:523.

Harrison, S. C., Olson, A. J., Schutt, C. E., Winkler, F. K., and Bricogne, G., 1978, Tomato bushy stunt virus at 2.9 Å resolution, *Nature (London)* **276**:368.

Henriksson, D., Tanis, R. J., Hawett-Emett, D., Tashian, R. E., and Nyman, P. O., 1980, *J. Mol. Biol.* (in press).

Henry, C. J., Slifkin, M., Merkow, L. D., and Pardo, M., 1971, The ultrastructure and nature of adenovirus type 2 induced paracrystalline formations, *Virology* **44**:215.

Holmes, K. C., 1980, Protein–RNA interactions during the assembly of tobacco mosaic virus, *Trends Biochem. Sci.*, January, p. 4.

Holmes, K. C., Stubbs, G. J., Mandelkow, E., and Gallwitz, V., 1975, Structure of tobacco mosaic virus at 6.7 Å resolution, *Nature (London)* **254**:192.

Hsu, C. H., White, J. A., and Sehgal, O. P., 1977, Assembly of southern bean mosaic virus from its two subviral intermediates, *Virology* **81**:471.

Huxley, H. E., and Brown, W., 1967, The low X-ray diagram of vertebrate striated muscle ands its behaviour during contraction and rigor, *J. Mol. Biol.* **30**:383.

Ibel, K., 1976, The neutron small-angle camera D11 at the high-flux reactor, Grenoble, *J. Appl. Crystallogr.* **9**:296.

Incardona, N. L., and Kaesberg, P., 1964, A pH induced structure change in bromegrass mosaic virus, *Biophys. J.* **4**:11.

Jack, A., 1973, Direct determination of X-ray phases for tobacco mosaic virus protein using non-crystallographic symmetry, *Acta Crystallogr. Sect. A* **29**:545.

Jack, A., and Harrison, S. C., 1975, On the interpretation of small-angle X-ray solution scattering from spherical viruses, *J. Mol. Biol.* **99**:15.

Jack, A., Harrison, S. C., and Crowther, R. A., 1975, Structure of tomato bushy stunt

virus. II. Comparison of results obtained by electron microscopy and X-ray diffraction, *J. Mol. Biol.* **97**:163.

Jacrot, B., 1976, The study of biological structures by neutron scattering from solution, *Rep. Prog. Phys.* **39**:911.

Jacrot, B., and Zaccai, G., 1980, The determination of molecular weights by neutron scattering, *Biopolymers* (in press).

Jacrot, B., Pfeiffer, P., and Witz, J., 1976, The structure of a spherical plant virus (browngrass mosaic virus) established by neutron diffraction, *Philos. Trans. R. Soc. London Ser. B* **276**:109.

Jacrot, B., Chauvin, C., and Witz, J., 1977, Comparative neutron small-angle scattering study of small spherical RNA viruses, *Nature (London)* **266**:417.

Johnson, J. E., Rossmann, M. G., Smiley, I. E., and Wagner, M. A., 1974, Single crystal X-ray diffraction studies of southern bean mosaic virus, *J. Ultrastructure. Res.* **46**:441.

Johnson, J. E., Akimoto, T., Suck, D., Rayment, I., and Rossmann, M. G., 1976, The structure of southern bean mosaic virus at 22.5 Å resolution, *Virology* **75**:394.

Kaper, J. M., 1975, *The Chemical Basis of Virus Structure, Dissociation and Reassembly*, North-Holland, Amsterdam.

Klug, A., and Caspar, D. L. D., 1960, The structure of small viruses, *Adv. Virus Res.* **7**:225.

Klug, A., Crick, F. H. C., and Wyckoff, H. W., 1958, Diffraction by helical structures, *Acta Crystallogr.* **2**:199.

Klug, A., Longley, W., and Leberman, R., 1966, Arrangement of protein subunits and the distribution of nucleic acid in turnip yellow mosaic virus, *J. Mol. Biol.* **15**:315.

Kneale, G. G., Baldwin, J. P., and Bradbury, E. M., 1977, Neutron scattering studies of biological macromolecules in solution, *Q. Rev. Biophys.* **10**:485.

Kratky, O., and Pilz, I., 1972, Recent advances and applications of diffuse X-ray small-angle scattering on biopolymers in dilute solutions, *Q. Rev. Biophys.* **5**:481.

Luzzati, V., 1960, Interpretation des mesures absolues de diffusion centrale des rayons X en collimation ponctuelle ou linéaire: Solutions de particules globulaires et de batonnets, *Acta Crystallogr.* **13**:939.

Luzzati, V. Tardieu, A., Mateu, L., and Stuhrmann, H. B., 1976, Structure of human serum lipoproteins in solution. I. Theory and techniques of an X-ray scattering approach using solvents of variable density, *J. Mol. Biol.* **101**:115.

Makowski, L., and Caspar, L. D., 1978, Filamentous bacteriophage Pf1 has 27 subunits in its axial repeat, in: *single stranded DNA phages* (D. Ray and D. Denhart, eds.), pp. 627–643, Cold Spring Harbor Laboratories, Cold Spring Harbor, N.Y.

Makowski, L., Caspar, D. L. D., and Marvin, D. A., 1980, Filamentous bacteriophage Pf1 structure determined at 7 Å resolution by refinement of models for the α helical subunit, *J. Mol. Biol.* **140**:149.

Marvin, D. A., 1966, X-ray diffraction and electron microscope studies on the structure of the small filamentous bacteriophage fd, *J. Mol. Biol.* **15**:8.

Marvin, D. A., and Hohn, B., 1969, Filamentous bacterial viruses, *Bacteriol. Rev.* **33**:172.

Marvin, D. A., and Wachtel, E. J., 1975, Structure and assembly of filamentous bacterial viruses, *Nature (London)* **253**:19.

Marvin, D. A., and Wachtel, E. J., 1976, Structure and assembly of filamentous bacterial viruses, *Philos. Trans. R. Soc. London Ser. B* **276**:81.

Marvin, D. A., Wiseman, R. L., and Wachtel, E. J., 1974, Filamentous bacterial viruses. XI. Molecular architecture of the class II (Pf1, xf) virion, *J. Mol. Biol.* **82**:121.

Phillips, J. C., Wlodawer, A., Yeviyz, M. M., and Hodgson, K. D., 1976, Applications of synchrotron radiation to protein crystallography: Preliminary results, *Proc. Natl. Acad. Sci. USA* **73**:128.

Rayment, I., Johnson, I. E., Suck, D., Akimoto, T., and Rossmann, M. G., 1978. An 11 Å resolution electron density map of southern bean mosaic virus, *Acta Crystallogr. Sect. B* **34**:567.

Rayment, I., Johnson, J. E., and Rossmann, G., 1979, Metal-free southern bean mosaic virus crystals, *J. Biol. Chem.* **254**:5243.

Rees, M. W., Short, M. N., and Kassanis, B., 1970, The amino acid composition, antigenicity and other characteristics of the satellite viruses of tobacco necrosis virus, *Virology* **40**:448.

Rossmann, M. G., Smiley, I. E., and Wagner, M. A., 1973. Crystalline cowpea chlorotic mottle virus, *J. Mol. Biol.* **74**:255.

Schneider, D., Zulauf, M., Schäfer, R., and Franklin, R. M., 1978, Structure and synthesis of a lipid-containing bacteriophage. XXVI. Neutron small angle scattering on bacteriophage PM2, *J. Mol. Biol.* **124**:97.

Schutt, C., and Winkler, F. W., 1977, in: *The Rotation Method in Crystallography* (V. W. Arndt and A. J. Wonacott, eds.), North-Holland, Amsterdam.

Serwer, P., 1977, Flattening and shrinkage of bacteriophage T7 after preparation for electron microscopy by negative staining, *J. Ultrastruct. Res.* **58**:235.

Sjöberg, B., 1977, A small angle X-ray investigation of the satellite of tobacco necrosis virus, *Eur. J. Biochem.* **81**:277.

Strandberg, B., Lentz, P., Kannan, K., Vaara, I., Unge, T., Fridborg, K., Borell, A., Petef, G., and Nordman, C., 1975, The structure of satellite tobacco necrosis virus: Three-dimensional X-ray diffraction analysis at 10 Å resolution, *Acta Crystalogr. Sect. A* **31**:S46.

Stubbs, G., Warren, S., and Holmes, K., 1977, Structure of RNA and RNA binding site in tobacco mosaic virus from 4 Å map calculated from X-ray fiber diagrams, *Nature* (*London*) **267**:216.

Stuhrmann, H. B., 1978, The use of X-ray synchroton radiation for structural research in biology, *Q. Rev. Biophys.* **11**:71.

Subirana, J. A., Lloveran, J., Lombardero, M., and Vinuela, E., 1979, X-ray scattering of the non-isometric bacillus subtilis phage ϕ 29, *J. Mol. Biol.* **128**:101.

Suck, D., Rayment, I., Johnson, J. E., and Rossmann, M. G., 1978, The structure of southern bean mosaic virus at 5 Å resolution, *Virology* **85**:187.

Tollin, P., Wilson, H. R., and Young, D. W., 1968, X-ray diffraction evidence of the helical structure of narcissus mosaic virus, *J. Mol. Biol.* **34**:189.

Torbet, J., and Maret, G., 1979, Fibers of highly oriented Pf1 bacteriophage produced in a story magnetic field, *J. Mol. Biol.* **134**:843.

Tremaine, J. M., 1966, The amino acid and nucleotide composition of the bean and cowpea strains of southern bean mosaic virus, *Virology* **30**:348.

Unge, T., and Strandberg, B., 1979, Iodination of satellite necrosis virus (STNV) for crystallographic structure determination, *Virology* **96**:80.

Unge, T., Liljas, L., Strandberg, B., Vaara, I., Kannan, K. K., Fridborg, K., Borell, A., Nordman, C. E., and Lentz, P., 1980, Satellite tobacco necrosis virus structure at 4.0 Å resolution, *Nature* **285**:373.

Veerisetty, V., 1978, Relationships among structural parameters of virions of helical symmetry, *Virology* **84**:523.

Watson, J. D., 1954, The structure of tobacco mosaic virus, I. X-ray evidence of a helical arrangement of sub-units around the longitudinal axis, *Biochem. Biophys. Acta* **13**:10.

White, R. A., and Fischbach, F. A., 1973, An X-ray scattering investigation of broad bean mottle virus in solutions of various electron densities, *J. Mol. Biol.* **75**:549.

Wilson, H. R., and Tollin, P., 1969, Some observations on the structure of potato virus X, *J. Gen. Virol.* **5**:151.

Wilson, H. R., Tollin, P., and Rahman, A., 1973, The structure of narcissus mosaic virus, *J. Gen. Virol.* **18**:181.

Winkler, F. K., Schutt, C. E., Harrison, S. C., and Bricogne, G., 1977, Tomato bushy stunt virus at 5.5 Å resolution, *Nature* (*London*) **265**:509.

Wiseman, R. L., and Day, L., 1977, Different packaging of DNA in the filamentous viruses Pf1 and Xf, *J. Mol. Biol.* **116**:607.

Ysabaert, M., van Emmelo, J., and Fiers, W. J., 1980, *J. Mol. Biol.* (in press).

Zipper, P., Kratky, D., Herrmann, R., and Hohn, T., 1971, An X-ray small angle study of the bacteriophages fr and R17, *Eur. J. Biochem.* **18**:1.

CHAPTER 4

Serological Methods in the Identification and Characterization of Viruses

M. H. V. Van Regenmortel

Laboratoire de Virologie
Institut de Biologie Moléculaire et Cellulaire
67000 Strasbourg, France

1. INTRODUCTION

The purpose of this chapter is to present an integrated view of the various serological techniques that have been used in virology. The accent will be placed on the principles that govern each type of test and on the general applicability of the different serological techniques in all fields of virus research. In recent years, advances in serological techniques have sometimes been applied in only one area of virology, although they could have been equally useful to workers studying other groups of viruses. No doubt this stems from the host-oriented approach that has guided the compartmentation of virology into separate fields of specialization. When it comes to serological properties, however, the similarities between animal, insect, bacterial, and plant viruses are paramount. The same immunochemical principles govern the *in vitro* serological reactions of all viral antigens, and much of general interest can be learned from the findings obtained with each particular group of viruses. An attempt will be made here to emphasize the general validity of specific experimental procedures. A number of recent reviews restricted to the serology of particular groups of viruses are available

(Cowan, 1973; Schmidt and Lennette, 1973; Ball, 1974; Kurstak and Morisset, 1974; Burns and Allison, 1975; Mazzone and Tignor, 1976; Mayr *et al.*, 1977; Tyrrell, 1978; Van Regenmortel, 1978; Cooper, 1979).

2. REAGENTS

2.1. Viral Antigens

It is beyond the scope of this chapter to consider the various purification methods that have been used to obtain viruses and viral soluble antigens suitable for antiserum production and serological analysis. The reader is referred to standard textbooks of virology and to some specialized reviews (Van Regenmortel, 1966; Francki, 1972; Appleyard and Zwartouw, 1978).

Methods of protein purification that are particularly helpful for purifying viruses are precipitation with polyethylene glycol (Juckes, 1971; Polson *et al.*, 1972; Venekamp, 1972) and separation by electrophoresis (Polson and Russell, 1967; Van Regenmortel, 1964, 1972). Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate and crossed immunoelectrophoresis have been used successfully to obtain pure protein subunits of viral capsids. The band corresponding to the component of interest can be cut out of the gel and used as such for immunization (Vestergaard, 1975; Carroll *et al.*, 1978).

The degree of purity of the antigen that is required in individual cases is extremely variable. When used as a diagnostic reagent, it is important that the antigen should be free of contaminating viruses and nonspecific inhibitors that could interfere in the serological tests (Schmidt and Lennette, 1971; Polley, 1977). For the purpose of antigenic analysis, it is important that host-specific antigens should be absent (Van Regenmortel, 1963). These contaminants can be removed by the use of antihost serum and specific immunoadsorbents (Gold, 1961; Avrameas *et al.*, 1969; Birkbeck and Stephen, 1970; Sugiura and Nakajima, 1977; Polson *et al.*, 1978).

2.2. Antisera

Rabbits are commonly used for producing antisera against viral antigens, although goats and sheep are sometimes employed when large quantities of antiserum are needed. Ascitic fluid from immunized mice

is also a convenient source of viral antibody (Sartorelli *et al.*, 1966; Kiriyama and Ohsumi, 1973; Tzianabos *et al.*, 1976).

Little reliable information is available regarding the relative merits of different immunization procedures. In rabbits, the antiviral immune response measured in individual animals submitted to the same immunizing procedure was found to be highly variable (Van Regenmortel and Von Wechmar, 1970). As a result, comparative trials designed to demonstrate the superiority of a particular method are likely to be extremely onerous and, in fact, very few such comparative studies have been reported. One generalization that appears to be valid is that the use of adjuvants is advantageous. A variety of empirical procedures that give satisfactory results have been described in several reviews of immunization practices (Chase, 1967; Horwitz and Scharff, 1969*a*; Crowle, 1973; Herbert, 1978).

It seems that many workers do not appreciate the extent to which individual antisera obtained from a series of bleedings of different animals can vary, both quantitatively and qualitatively. It cannot be overemphasized that it is essential to immunize a number of animals and to take several bleedings from each one, whatever the purpose of the serological study may be. For the same reason, the practice of indiscriminately pooling antisera can be detrimental, since it could reduce the potency of an individual antiserum which possesses the desired characteristics.

The use of inbred strains of animals for decreasing the heterogeneity of the immune response has received little attention from virologists. On the other hand, the recently developed method of cell fusion of malignant cells and antibody-producing cells (Köhler and Milstein, 1975) is being applied successfully for producing homogeneous antiviral antibodies. This method, which is based on the production of somatic cell hybrids between mouse myeloma cells and spleen cells derived from mice immunized with viruses, holds tremendous promise for the elucidation of various aspects of antiviral immunity. Monoclonal antibodies against influenza and rabies viruses have been obtained from such hybridomas (Gerhard *et al.*, 1978; Wiktor and Koprowski, 1978). One interesting finding obtained from such studies is that monoclonal antibodies expressed by hybridomas appear to lack the usual cross-reactivity between virus strains and variants that is found with antibodies from normal mice. The absence of this cross-reactivity may be due to the fact that, in normal immunized mice, only a small population of splenic cells is able to recognize identical determinants in the two strains. If most clones of splenic cells do in fact express antibody specificities unique to the immunizing variant, it is

normal that most hybridoma antibodies do not recognize altered antigenic determinants in the heterologous variants. There is some evidence that monoclonal antibodies are not able to recognize a complementary antigenic determinant when it has been altered in a variant at a single amino acid position (Laver, personal communication). These preliminary results make it clear that hybridoma antibodies represent a powerful new tool for investigating viral antigens.

3. NEUTRALIZATION

Virus neutralization tests measure the loss of virus infectivity resulting from the binding of antibody molecules to the surface of virions. The mechanisms underlying this decrease in infectivity are complex and have not yet been fully elucidated. Clearly, the antibody interferes with an early stage of the infectious process, i.e., with adsorption to the host cell, penetration, or uncoating of the virions, but the precise step which is blocked may not be the same with all viruses. The sensitivity of the host cell used for the infectivity assay and the route of inoculation may also play a role. Different levels of residual infectivity may be observed when the same virus–antibody complex is assayed in different host cells (Philipson, 1966), and higher antibody titers are often obtained in less sensitive hosts.

Virus infectivity can be measured by any available titration method, using animals, embryonated eggs, monolayer cell cultures in tubes or microtiter plates, plaque assays, mechanical inoculation onto leaf surfaces, or inhibition of vector transmissibility. Since, with plant viruses, quantitative infectivity assays are neither sensitive nor very accurate, neutralization tests have rarely been used in plant virology.

Although neutralization tests generally are the most specific of all virus–antibody reactions, it must be realized that they do not provide information on the total antiviral antibody response. These tests obviously will not detect antibodies reacting with viral antigenic structures that are not directly involved in the expression of infectivity. Neutralization of influenza virus is produced only by antibodies directed against the hemagglutinin component of the membrane and not by those directed against the neuraminidase (Webster and Laver, 1967) or the matrix protein (Oxford and Schild, 1976). In the case of foot-and-mouth disease virus, the antibody-binding site present on the face of the icosahedral particle (Brown and Smale, 1970) is not involved in neutralization, whereas the two sites present on the vertices are (Rowlands *et al.*, 1971). In the case of vesicular stomatitis virus, which is com-

posed of five polypeptide chains and RNA, antisera to the virus contain antibodies to the nucleoprotein core antigen (Cartwright *et al.*, 1970) and to the spike glycoprotein. It was found that neutralizing activity of antiserum could be fully inhibited by the purified glycoprotein, which suggests that it is only the latter component which is involved in the neutralization reaction (Kelley *et al.*, 1972).

The distribution of different antigens over the surface of adenoviruses is also well established (Pettersson, 1971; Wadell, 1972; Willcox and Mautner, 1976*a*,*b*). Antisera were prepared against isolated hexons, pentons, and fibers, and were tested for neutralizing activity. By far the most efficient neutralization was obtained with antihexon antibodies. Antibodies against penton bases and fibers produced only negligible neutralization when used separately, although considerable neutralization occurred when both antisera were used together (Norrby and Wadell, 1972).

Another problem is that the extent of neutralization is very much dependent on the "quality" of the antibody, i.e.. on such factors as antibody affinity and valence. This means that neutralization assays may be unreliable for comparing the amounts of antibody present in different antisera (Haimovich and Sela, 1966; Blank *et al.*, 1972). In spite of these limitations, however, neutralization tests have been found invaluable for diagnostic and epidemiological studies, and they have contributed more than any other technique to our understanding of the interaction between virus and antibody. A number of reviews dealing with various aspects of virus neutralization are available (Fazekas de St. Groth, 1962; Svehag, 1968; Osterrieth, 1972; Daniels, 1975; Mandel, 1979).

3.1. Mechanism of Neutralization

The mechanism of neutralization has been investigated mainly by following the kinetics of the neutralization reaction. The procedure consists of mixing antibody with virus and removing, at regular time intervals, an aliquot of the mixture, which is then assayed for infectivity. When the percentage of surviving virus is plotted against time, an initial linear exponential decline in virus infectivity is usually observed (Dulbecco *et al.*, 1956; Granoff, 1965). Such apparent first-order kinetics are commonly attributed to the fact that a single hit results in virus neutralization (see Svehag, 1968). According to Daniels (1975), however, these data do not establish that one antibody molecule alone will neutralize an infectious particle. This author suggests that the

attachment of a single antibody may be the terminal event corresponding to the neutralization of an infectious virus–antibody complex formed in the early stages of the reaction (i.e., within a few seconds).

In many systems, deviations from a linear exponential decline, for instance, as an initial lag phase, have been observed (Lafferty, 1963). Furthermore, in the later stages of the reaction, a point is reached when no further decrease in infectivity occurs. The residual infective virions are not genetic variants, because their progeny are inactivated at the same rate as the original virus population. This residual infectivity, called the persistent fraction, has been ascribed to a variety of causes:

1. Aggregation of virus particles, which prevents virions in the center of the aggregate from coming into contact with antibody (Wallis and Melnick, 1967, 1970; Hahon, 1970).
2. Steric hindrance by neighboring antibodies, which prevents critical sites on the virion from being reached (Lafferty, 1963; Ashe *et al.*, 1969; Rappaport, 1970).
3. Dissociation of virus–antibody complexes (Fazekas de St. Groth and Webster, 1963).

Although each explanation is supported by some experimental evidence, other data again show that, in many systems, the hypothesis of aggregation (Ashe *et al.*, 1969; Hahon, 1970) or of dissociation (Mandel, 1961) is not tenable.

Most investigators have been unable to demonstrate any significant reversibility of the virus–antibody reaction by dilution of the reaction mixture (see Svehag, 1968). This may be due to the low dissociation constant of the reaction which may necessitate intervals of several days to produce detectable dissociation. Another reason may be that, after an initial reversible reaction involving only one of the two IgG combining sites, a secondary reaction occurs whereby the second antibody site binds to a neighboring antigenic group on the virion (Lafferty and Oertelis, 1963). Such monogamous bivalent binding can occur because IgG molecules are sufficiently flexible to adjust their two identical combining sites to fit neighboring subunits on the viral surface. When such a complex is formed, the free energy change is much greater than when the antibody molecule binds by only one site (Keller, 1966; Klinman *et al.*, 1967; Hornick and Karush, 1972). With tobacco mosaic virus (TMV) and IgG antibody, it was found that no monogamous bivalent binding occurred in extreme antibody excess (Van Regenmortel and Hardie, 1976). On the other hand, when the same excess of antibody was added stepwise by a series of successive small increments, bivalent binding occurred exclusively. The more rapid

univalent binding seems to be favored when the antibody is added all at once, and this allows exactly twice as many antibody molecules to attach to the virus surface than when antibody is added more slowly (Van Regenmortel and Hardie, 1976). The energetically favored bivalent binding of antibody may thus be restricted only to conditions of antigen excess.

In the case of the tailed bacteriophages, most of the phage-neutralizing activity of antibodies appears to be directed against the tail fibers of baseplates (Franklin, 1961; King, 1968; Berget and King, 1978). It has been suggested that the binding of antibodies to the tail fibers causes some of the fibers to adopt a coiled conformation (Stemke *et al.*, 1974), and that antibody directed against the phage whiskers in the collar region immobilizes the fibers in a restricted position (Conley and Wood, 1975).

Recently, still another mechanism which applies to viruses that can undergo reversible conformational transitions has been proposed, to explain both neutralization and its apparent one-hit nature (Mandel, 1976). The capsid of poliovirus has been shown to undergo a transition between two electrophoretically recognizable states, A and B, characterized by isoelectric points of about 7 and 4.5, respectively (Mandel, 1971). When virus reacts with antibody, it becomes stabilized in state B without producing any particles of intermediate isoelectric point. It has been suggested that the virion is infectious only in state A and that the reaction of a single antibody molecule with one antigenic determinant causes the viral subunit containing this determinant to be stabilized in the B conformation. Through cooperative conformational transitions, all subunits would then become stabilized in the same state, and the altered virion would be resistant to uncoating (Mandel, 1976).

In recent years, much evidence favoring the multihit model of virus neutralization has accumulated (see Daniels, 1975; Della-Porta and Westaway, 1978). For instance, the synergistic effect of mixtures of antibody molecules directed at the different antigenic determinants of adenoviruses clearly contradicts the single-hit model of neutralization (Hierholzer and Dowdle, 1970; Norrby and Wadell, 1972). Similarly, results obtained with phenotypically mixed virus particles derived by mixed infection with two serologically distinguishable viruses show that greatly enhanced neutralization is achieved by the combined action of antibodies directed against the two sets of viral antigens (Choppin and Compans, 1970).

Further data obtained with flaviviruses and which are also incompatible with a single-hit model have been reviewed by Della-Porta and Westaway (1978). According to these authors, the "critical areas" at

the surface of virions that need to be covered by antibody to achieve neutralization are too large to allow inactivation by single immunoglobulin molecules.

On the other hand, in two recent papers (Trautman, 1976; Trautman and Harris, 1977), it has been argued that data suggesting one-hit neutralization kinetics can be reconciled with the presence of as many as five to ten critical sites on a small particle such as foot-and-mouth disease virus. Using the mass-action theory and a computer simulation approach, it was found that three of these critical sites had to be free for the virus to be infectious. Trautman (1976) derived an explicit expression for the extent of reaction between virus and antibody which allows the computer simulation of known systems. The utilization of this approach in the future should no doubt contribute to the elucidation of the mechanisms of virus neutralization.

3.2. Virus Sensitization

Virus sensitization refers to a situation where antibodies attached to the surface of virions do not neutralize the infectivity but "sensitize" the virus to subsequent inactivation by a third component such as antiglobulin, complement, or staphylococcal protein A (Notkins, 1971; Majer, 1972; Takabayashi and McIntosh, 1973; Austin and Daniels, 1974). Although virus–antibody complexes in the persistent fraction may contain sensitized virus (Majer and Link, 1970), the phenomenon of sensitization is not restricted to the non-neutralizable fraction. Antibodies directed to antigenic structures that are not involved in the expression of infectivity, such as the neuraminidase of influenza virus (Webster and Laver, 1967), are able to sensitize viruses. This means that the sensitization reaction is a useful tool for studying viral antigens that do not react with neutralizing antibody.

Neutralization of sensitized virus by antiglobulin antibodies has been found to be a sensitive method for detecting minute amounts of antiviral antibody (Goodman and Donch, 1965), for studying cross-reactions between viruses (Kjellen and Pereira, 1968), and for titrating various antiglobulin preparations (Daniels *et al.*, 1970). Certain rheumatoid factors such as IgM molecules with antiglobulin activity also bind to sensitized virus, but, in this case, neutralization is only achieved by the subsequent action of complement (Ashe *et al.*, 1971; Gipson *et al.*, 1974). It has been suggested that the inability of rheumatoid factor by itself to neutralize sensitized virus is due to the

large size of the IgM molecule which may prevent effective covering of critical sites.

Sensitization by complement components has been studied with numerous enveloped viruses, for instance, with herpesvirus (Yoshino and Taniguchi, 1965), Newcastle disease virus (Linscott and Levinson, 1969), and equine arteritis virus (Radwan and Burger, 1973). The early-acting complement components (C1 to C4) appear to neutralize infectivity through a steric hindrance phenomenon resulting from the packing of bound molecules at the surface of the virion (Radwin and Crawford, 1974). The terminal complement components (C5 to C9), on the other hand, act on the viral membrane by inducing lysis of virus particles and loss of the internal components (Oroszlan and Gilden, 1970; Daniels, 1975). The mechanism of virolysis by the action of complement is similar to the destruction of the cell membrane in immune cytolysis. Both processes result from the enzymatic breakdown of a lipoprotein membrane and can be visualized, in the electron microscope, in the form of similar lesions in the virus envelopes and cell membranes (Berry and Almeida, 1968; Oldstone, 1975). Non-membrane-containing viruses such as polyoma virus are resistant to lysis by complement even in the presence of DNase (Oldstone *et al.*, 1974).

Instead of protecting the host against unwanted infections, the interaction of complement with virus–antibody complexes *in vivo* may lead sometimes to a virus-induced immune complex disease. In persistent virus infections, the continuous antigenic challenge can lead to the formation of virus–antibody complexes that become trapped in various tissues of the host. Granular deposits of virus, host immunoglobulin, and complement in renal glomeruli and arteries have been demonstrated with numerous chronic viral infections and appear to be a common cause of immune complex disease (Oldstone, 1975).

3.3. Methods

3.3.1. Classical Neutralization Test

The classical neutralization test is performed either by incubating a series of antiserum dilutions with a constant amount of virus or, alternatively, by incubating a constant amount of antiserum with increasing dilutions of virus. The first method is more economical in the use of antiserum and is more generally used with tissue culture indicator systems. The second method is preferable when antisera have a low

antibody content. Following incubation, the infectivity of the virus can be tested in various indicator systems such as monolayer tube cultures or embryonated eggs. Detailed experimental procedures are described by Casals (1967), Habel (1969), Lennette and Schmidt (1969), Rovozzo and Burke (1973), and Mayr *et al.* (1977).

3.3.2. Microneutralization Tests

Following the development of disposable plastic microtiter plates, microneutralization tests suitable for numerous viruses have been described (Kenny *et al.*, 1970; Schmidt and Lennette, 1973). The results can be scored in terms of the suppression of a viral cytopathic effect or by utilizing the phenomenon of hemadsorption which can be brought into play by adding certain erythrocytes to the plastic cups (Sorensen, 1974; Wooley *et al.*, 1974; Greig, 1975). Results can also be evaluated colorimetrically by observing the color change of an indicator added to the growth medium (Witte, 1971). This follows from the fact that acid production which normally accompanies the growth of cells is suppressed by virus infection.

3.3.3. Plaque Reduction Test

Initially, the plaque reduction test was restricted to the study of bacteriophage neutralization, and the results were scored by the standard double agar layer method of phage titration (Adams, 1959). The assay of neutralized phage was improved by the "decision" technique of Jerne and Avegno (1956), and by the complex inactivation method, which uses an antiglobulin serum (Goodman and Donch, 1965; Krummel and Uhr, 1969). Following the development of plaque assays for animal viruses (Dulbecco and Vogt, 1954), the plaque reduction test has become increasingly used with many viruses (Fiala, 1969; Schmidt and Lennette, 1973; McVicar *et al.*, 1974). The foci of virus-infected cells (plaques) are made more easily visible by the staining of the background viable cells with vital stains.

3.3.4. Neutralization Kinetics Test

The sensitive neutralization kinetics test which measures the speed of virus neutralization by antibodies is particularly suited for determin-

ing small antigenic differences between closely related virus strains. A neutralization rate constant is determined which is normalized by expressing the results obtained in a heterologous test as a percentage of the rate constant observed in the homologous system (McBride, 1959). This method has been used in strain differentiation with herpes simplex virus (Ashe and Scherp, 1963; Wheeler *et al.*, 1969), rhinovirus (Cooney *et al.*, 1973), reovirus (Munro and Wooley, 1973), poliovirus (McBride, 1959; Thouvenot *et al.*, 1973), vaccinia virus (Dunlap and Barker, 1973), and bovine rhinotracheitis virus (Potgieter and Maré, 1974). In a recent study of foot-and-mouth disease virus strains, the method was compared with the plaque reduction and microneutralization tests and found to allow a similar degree of differentiation between strains (Rweyemamu *et al.*, 1977). In contrast, the complement fixation test was less capable of distinguishing between different strains.

3.3.5. Reactivation of Neutralized Virus

The neutralization of viruses by homologous antibody in the body fluids sometimes hampers the detection of inapparent viral infections. This is the case with Newcastle disease virus in poultry, where inapparent infections in convalescent or vaccinated birds are believed to play a role in the dissemination of the disease (Gillette *et al.*, 1975). The usual virus isolation procedures generally fail to detect these infections, and it is necessary, for efficient recovery of the virus, first to dissociate the virus–antibody complexes. Neutralized Newcastle disease virus was efficiently reactivated by proteolytic digestion with trypsin or papain (Brugh, 1977). Poliovirus and foot-and-mouth disease virus have been reactivated by treatment with fluorocarbon (Brown and Cartwright, 1960; Svehag, 1963). Other reactivation methods that have been used successfully are sonic treatment (Keller, 1965) and the use of pH extremes (Mandel, 1961; Granoff, 1965; Kjellén, 1966).

3.3.6. Serological Blocking of Aphid Transmission

This infectivity neutralization test is assayed by determining whether aphids fed on virus–antibody mixtures can transmit the virus to healthy plants (Gold and Duffus, 1967; Duffus and Gold, 1969). The method can be used to demonstrate serological relationships between plant viruses when the usual in vitro methods cannot be applied, for instance, with viruses that are not mechanically transmissible (Rochow

and Ball, 1967). This method has been of considerable help in clarifying the relationships between members of the luteovirus group (Duffus and Russell, 1975; Rochow and Duffus, 1978).

4. AGGLUTINATION

It is customary to distinguish between agglutination and precipitation reactions on the basis of the size of the reacting antigen. The term "precipitation" is used to describe the insolubilization of virus particles through the action of antibody, whereas the term "agglutination" refers to the clumping of cells or of particles of similar size. Fewer antibody molecules are needed to produce an agglutination visible to the naked eye than are required to produce a visible precipitate. This has led to the development of numerous procedures which extend the range of visual detection of serological reactions by means of reagents such as red blood cells and latex particles coated with antibody or viral antigens.

4.1. Viral Hemagglutination

Many viruses are able to bind to receptors present on the surface of red blood cells of certain animal species. This leads to the agglutination of the erythrocytes, a phenomenon called hemagglutination. Since hemagglutination does not take place when the surface of a virion is covered with antibody molecules, it is possible to use hemagglutination inhibition tests to assay for the presence of viral antibodies.

The components of the virus surface responsible for the hemagglutinating property are known as hemagglutinins and consist usually of glycoproteins or lipoproteins. In the case of adenoviruses, the hemagglutinating activity resides in the fiber component. Soluble hemagglutinins are often present in viral extracts, either because they are released from intact virions or because they are produced in the infected cell in large excess compared to the intact virions.

Soluble hemagglutinins that are monovalent with respect to their binding sites for erythrocytes cannot agglutinate cells. Although they do combine with the cell receptors, they produce agglutination only after becoming linked following the addition of antihemagglutinin antibodies (Norrby, 1968).

The best-studied hemagglutinins are those of myxoviruses, and a number of reviews describing their structural and functional properties

are available (Howe and Lee, 1972; Schulze, 1973; Laver, 1973). Much of the interest in the influenza hemagglutinin is due to the fact that it undergoes wide antigenic variation and that this phenomenon is responsible for the continual outbreaks of new influenza epidemics (Webster and Laver, 1975). Two distinct types of antigenic variation have been shown to occur. The first is antigenic drift, which involves gradual changes in the amino acid sequence of the hemagglutinin polypeptide. The second type of variation consists of major antigenic shifts which are caused by sudden, vast differences in amino acid sequence which cannot be explained by mutation. It is thought that such "new" human viruses are formed as a result of genetic recombination between "old" human viruses and animal influenza virus strains.

A phenomenon related to viral hemagglutination is hemadsorption, which occurs with those viruses that possess hemagglutinins and are released from the host cell by a process of budding. During the release of the virus at the cell surface, the infected cells are able to adsorb erythrocytes. In hemadsorption inhibition, the infected cells are treated with viral antiserum prior to the addition of red cell suspensions (Schmidt and Lennette, 1973; Norrby *et al.*, 1977).

4.2. Hemagglutination Inhibition Tests

Hemagglutination inhibition tests may be used either to identify virus isolates by means of specific antisera or to measure antibody levels by means of standard virus suspensions. They are usually somewhat less sensitive than neutralization tests for detecting viral antibodies, but they are easier to perform and take less time to complete. However, hemagglutination inhibition tests are complicated by the presence, in many sera, of various nonspecific inhibitors of agglutination. Some of these nonspecific inhibitors block agglutinating sites on the erythrocytes, while others react with the antigen itself. It is essential to remove these inhibitors, but, unfortunately, no single, universally applicable method can be recommended. Good results in eliminating the inhibitors have been obtained by treating antiserum with ether (Tauraso *et al.*, 1971), kaolin (Monath *et al.*, 1970; Inouye and Kono, 1972), dextran sulfate and $CaCl_2$ (Liebhaber, 1970; Nelson *et al.*, 1972), rivanol (Toms and Mostratos, 1973), and DEAE-Sephadex (Altemeyer *et al.*, 1970).

In addition to the problem of nonspecific inhibitors, it has also been reported (Reno and Hoffman, 1972) that certain components of

complement can enhance the hemagglutination inhibition caused by specific antibody.

In recent years, hemagglutination has been shown to occur with many more virus–erythrocyte combinations than previously known, and useful diagnostic tests are now available for most human and animal viruses, e.g., adenoviruses, enteroviruses, myxoviruses, reoviruses, and rhabdoviruses (Schmidt and Lennette, 1973). In some cases, it may be advantageous to use formalinized or glutaraldehyde-fixed erythrocytes, since they can be stored for several months; this eliminates the variability which is sometimes found in individual red blood cell preparations (Gupta and Harley, 1970; Wolff *et al.*, 1977). Freeze-dried, formalinized erythrocytes have been used in the detection of rubella antibodies (Van Weemen and Kacaki, 1976).

During the last few years, further advances have occurred in the testing of coronaviruses (Bingham *et al.*, 1975), certain herpesviruses (Klingeborn and Dinter, 1973), parvoviruses (Joo *et al.*, 1976), retroviruses (Witter *et al.*, 1973), rubella virus (Quirin *et al.*, 1972; Iwakata *et al.*, 1974), togaviruses (Della-Porta and Westaway, 1972), and papovaviruses (Favre *et al.*, 1974).

Detailed procedures for hemagglutination inhibition tests are described in Rosen (1969), Lennette and Schmidt (1969), Grist *et al.* (1974), Mayr *et al.* (1977), and Tyrrell (1978).

4.3. Passive Hemagglutination

Passive hemagglutination, also called indirect hemagglutination, utilizes erythrocytes to which virus particles have been coupled by various chemical treatments. The cells are first stabilized, for instance, with tannic acid (Stavitsky, 1977), sulfosalicyl acid (Becht, 1968), or glutaraldehyde (Becht and Malole, 1975). Bisdiazobenzidine (Arquilla, 1977) and carbodiimides were used successfully as coupling reagents. These antigen-coated red blood cells can be agglutinated by specific viral antibody, and the test is thus applicable in principle to any virus, not only to those which possess hemagglutinins. The passive hemagglutination reaction can be inhibited by prior incubation of the test serum with a suspension of the homologous virus. Such an inhibition lends itself to the measurement of very small amounts of viral antigen.

In view of the very wide applicability of direct viral hemagglutination procedures with animal viruses, passive hemagglutination tests have been used only occasionally in animal virology. Nevertheless, good

results have been reported, for instance, with herpesvirus (Bernstein and Stewart, 1971), rhinovirus (Faulk *et al.*, 1971), a murine leukemia virus (Sibal *et al.*, 1971), foot-and-mouth disease virus (Warrington and Kawakami, 1972), and the Australia antigen associated with viral hepatitis (Vyas and Shulman, 1970; Hollinger *et al.*, 1971).

In plant virology, passive hemagglutination has been used for detecting very small amounts of virus in crude and clarified plant sap (Cunningham *et al.*, 1966; Abu Salih *et al.*, 1968*a*). Red blood cells were coated with either viral antigen or antibody. Antiserum titers were determined with virus-coated erythrocytes or, in inhibition tests, with antibody-coated erythrocytes. Compared with the classical precipitin test in tubes, a thousandfold increase in serum titer can be obtained (Richter, 1967). It is also possible to detect very small amounts of virus, either with red cells sensitized with an optimal quantity of antibody or in inhibition tests using virus-coated erythrocytes. Such tests were found to be about 100–500 times more sensitive than tube precipitin tests in the detection of various elongated and isometric plant viruses (Saito and Iwata, 1964; Richter, 1971).

The sensitivity of the passive hemagglutination technique makes it well suited for studying weak cross-reactions between related viruses (Gamez *et al.*, 1967) and for demonstrating the weak serological activity of short peptides that correspond to certain antigenic determinants of a viral protein. This approach has been used in studies of the antigenic determinants of the coat protein of tobacco mosaic virus (TMV). The *C*-terminal hexapeptide of TMV protein was synthesized and coupled to erythrocytes, and passive hemagglutination tests were used to detect the presence, in TMV antisera, of antibodies reactive with the hexapeptide (Anderer and Ströbel, 1972*a*,*b*).

4.4. Latex Test

In the latex test, antigen or antibody is adsorbed onto commercially available polystyrene latex particles (Bactolatex, Difco Laboratories, Detroit, Michigan). The technique has been described in detail by Litwin (1977) and Bercks *et al.* (1972). Optimal sensitization of the latex is obtained by carefully selecting the dilution of antiserum or purified globulin preparation used for coating the particles (Bercks, 1967; Bercks and Querfurth, 1969). Different antisera should be tested since not all antisera are equally effective in this procedure.

Using antibody-coated latex, it is possible to detect 100- to 1000-

fold smaller quantities of virus than is possible with tube precipitin tests. This method has been used in the routine detection of numerous elongated and isometric plant viruses in crude extracts (Abu Salih *et al.*, 1968*b*; Maat, 1970; Schade, 1971; Fuchs, 1976; Koenig and Bode, 1978), as well as with insect and animal viruses (Fritz and Rivers, 1972; Carter, 1973).

It is possible to sensitize latex particles with viral antigens. The best results are obtained when an excess of antigen is adsorbed onto latex via an intermediate layer of previously adsorbed antibodies. This method has been used to study distant relationships within the potexvirus and tymovirus groups (Bercks and Querfurth, 1971). Homologous antiserum titers showed a maximal increase of 300-fold over the titers obtained in precipitin tests. Large increases in heterologous titers were obtained only when purified globulin fractions were used instead of unfractionated antisera.

5. PRECIPITATION

5.1. Quantitative Precipitin Tests in Tubes

Quantitative precipitin tests in fluid medium have been used mainly in plant virology because they require relatively large quantities of reactants. Purified antigen preparations have to be used since the tests cannot resolve multiple antigen–antibody systems. The tests are usually performed in tubes by mixing 0.5-ml volumes of suitable dilutions of antiserum and antigen, and determining the highest dilution of antiserum that will give a visible precipitate (i.e., the antiserum titer).

The various factors which influence the results of tube precipitin tests have been discussed in detail by Matthews (1967). One important point is that the size of the reacting antigen very much influences the antiserum titer. If was shown by Kleczkowski (1966) that about 10 times more antibody is required to precipitate a given amount of depolymerized tobacco mosaic virus protein than to precipitate the same amount of virus. Similarly, in the case of potato virus X and its protein subunits, Shepard and Shalla (1970) showed that the virus was a 25-times-better detector of a given amount of homologous antibody than the depolymerized protein. It is clear, therefore, that precipitin titers are not suitable for comparing antibody levels against antigens of different sizes. For the same reason, preparations of elongated viruses that have become aggregated during purification tend to give spuriously

high antiserum titers. The presence of small amounts of polyethylene glycol in the reaction mixture may also lead to higher precipitin titers (Wolf and Schmelzer, 1973).

Although it is widely believed that precipitation results from the formation of a lattice between large numbers of antigen and antibody molecules, this view appears to be too simplistic. In fact, precipitation does not occur in solutions of low ionic strength even when the reactants are present in optimal proportions and in spite of the fact that binding of antigen to antibody takes place normally in the absence of salt (Kleczkowski, 1965). Subsequent dissociation of antibody from the antigen, under acid conditions, is much facilitated when the initial binding is carried out in the absence of salt. This finding was used to develop an improved method for the isolation of specific viral antibody which possesses several advantages compared to standard procedures (Hardie and Van Regenmortel, 1977).

Tube precipitin tests have been found useful for quantifying the extent of serological cross-reaction between strains of elongated viruses, provided certain precautions are taken. It is well known that the extent of cross-reaction between two strains determined in reciprocal tests, i.e., when antisera are used against each of the two strains, can show considerable variation. Sometimes the extent of cross-reactivity is pronounced in one test and very weak or totally absent in the reciprocal test (Corbett, 1967; Van Regenmortel and Von Wechmar, 1970). However, when a sufficient number of bleedings obtained from different animals are used to compare average homologous and heterologous titers, reciprocal tests give very similar results. This allows the computation of a reliable index of serological cross-reactivity between virus strains (Van Regenmortel, 1975). The results obtained with numerous strains of tobacco mosaic virus indicate that it is possible to arrange them in a series of increasingly distantly related entities and that any sharp distinction between close and distant serological relationships is essentially arbitrary (Van Regenmortel, 1966, 1978). Similar conclusions have been drawn from serological studies of tymoviruses (Koenig and Givord, 1974; Koenig, 1976) and strains of tobacco necrosis virus (Uyemoto *et al.*, 1968).

5.2. Microprecipitin Tests in Droplets

Microprecipitin tests are performed in single drops of the mixed reactants deposited on the bottom of a petri dish. The drops are usually

covered with a layer of mineral oil to prevent drying out, and the reactions are observed by dark-field microscopy at 50–100 × magnification. Purified virus preparations as well as clarified extracts of infected tissue can be utilized for the test. The method is economical in its use of antiserum and is fairly sensitive since small precipitates are easily detected under the microscope. Microprecipitin tests have been extensively used by plant virologists for indexing plants for the presence of virus; their numerous applications have been reviewed by Van Slogteren (1969) and Ball (1974).

5.3. Immunoprecipitation

The technique of immune precipitation is a useful method for isolating intracellular proteins of virus-infected cells. During their multiplication within host cells, viruses induce the synthesis of a variety of proteins, only some of which become part of the assembled virions. Serological precipitation has been found extremely useful for following the rate of synthesis of virus-specific antigens by means of pulse-chase experiments. Usually, the labeled proteins in the extract are allowed to react with rabbit antibody specific for the antigen under study, and the resulting soluble complexes are precipitated with a second antiserum against rabbit globulins. Alternatively, the labeled antigen can be isolated by coprecipitation after addition of large amounts of the purified cold antigen and specific antibody. These procedures have been described in detail by Horwitz and Scharff (1969*b*). It is also possible to replace the second antibody directed against rabbit globulins by staphylococcal protein A. This protein binds specifically to IgG and decreases the problem of nonspecific binding observed with double antibody systems (Goding, 1978).

The immune precipitates are usually dissolved in buffers containing urea or sodium dodecyl sulfate and are analyzed by polyacrylamide gel electrophoresis (Shanmugam *et al.*, 1972). The study of virus-specific translation by immune precipitation has been especially valuable for analyzing the replication of retroviruses, since, in this case, host protein synthesis is not inhibited by virus infection and alternative methods of analysis are thus more difficult to use (Vogt and Eisenman, 1973; Van Zaane *et al.*, 1975; Naso *et al.*, 1975). Immunoprecipitation has also been used to study the radioiodinated surface antigen of Friend murine leukemia virus (Ihle *et al.*, 1975).

5.4. Double Diffusion

In double diffusion techniques, antigen and antibody diffuse toward each other in a gel which initially contained neither of them. As diffusion progresses, the two reactants meet, and precipitation occurs along a line where serological optimal proportions are reached. This line remains stationary if the ratio of the initial concentrations of the reactants corresponds to the equivalence point. In this case, the position of the line is a reflection of the Stokes' radius of the antigen. If one of the reactants is initially present in excess of the other, the precipitin line will broaden and shift toward the reservoir with the less concentrated reactant. Thus the position of the line can also be used to estimate the concentration of one or other reactant.

Another outstanding advantage of double diffusion analysis is that complex mixtures of antigens can be resolved and that the minimum number of antigen–antibody systems present can be enumerated.

Numerous parameters, in addition to size and relative concentration, can influence the diffusion process and the precipitation patterns. Agarose gels have been found superior to agar for resolving precipitin lines formed by enteroviruses (Styk and Schmidt, 1968). The electrolyte concentration in the gel (Wetter, 1967) as well as the kind of buffer used (Von Wechmar and Van Regenmortel, 1968) can markedly affect the formation of precipitin lines. The type of preservative used for keeping the gel free of microbial contamination may also be important. Mercury-containing preservatives which can degrade certain viruses are now mostly replaced by sodium azide (Cowan, 1966; Koenig, 1970).

It is generally accepted that, in a balanced immunological system, a single homogeneous antigen cannot give rise to more than one precipitin line. If artifacts arising from temperature variations or unsuspected contaminants can be excluded, the formation of double bands by apparently single systems has usually been attributed to the fact that the antigen was degraded into fragments with different serological specificities. It is unlikely, however, that this explanation applies to the two bands commonly observed with the protein subunit of tobacco mosaic virus (Kleczkowski, 1961; Van Regenmortel and Lelarge, 1973). When this subunit is isolated in the monomeric state from a single band in SDS polyacrylamide gels, it still gives rise to double precipitin lines. A possible explanation for this phenomenon is that antibody binding can induce, in the monomer, a conformational change similar to that observed during the polymerization of the subunit into capsids. Antibodies specific for each conformational state are known to be present in

most antisera (Van Regenmortel and Lelarge, 1973), and it is possible that they induce mutually exclusive surface determinants in the monomer.

Exhaustive reviews of all aspects of immunodiffusion techniques are available (Ouchterlony, 1968; Crowle, 1973; Ouchterlony and Nilsson, 1978).

5.4.1. Double Diffusion in Tubes

Double diffusion in tubes is especially valuable for measuring antigen and antibody concentration and for estimating the size of a viral antigen by means of its diffusion coefficient (Van Regenmortel, 1959). The location of a precipitin band in the tube is a linear function of the logarithm of the ratio of the concentration of the reactants and can be measured very accurately with a calibrated magnifying device (Polson, 1971). The applications of double diffusion tests in tubes for the study of viruses have been discussed by Van Regenmortel (1966).

5.4.2. Double Diffusion in Plates

Double diffusion in plates, often referred to as the Ouchterlony method, is one of the most widely used techniques in virus serology. In addition to its simplicity and economical use of reagents, its main advantage is that it is able to provide a visual demonstration of the relationships that exist between antigens. When antigens diffuse from neighboring wells in a gel toward a single antibody source, a variety of precipitation patterns can be observed. Patterns of coalescence, partial fusion, and crossing of precipitin lines reflect the presence or absence of common or related antigenic determinants on the different antigens. This type of study has been extensively used in all areas of virology for analyzing the antigenic relationship between groups of viruses (Van Regenmortel, 1966; Schmidt and Lennette, 1973; Brown *et al.*, 1973; Ball, 1974; Mazzone and Tignor, 1976; Moyer and Smith, 1977). The method has also been found invaluable for analyzing the antigenic properties of individual components of capsids, such as the hexon, penton, and fiber subunits of adenoviruses (Valentine and Pereira, 1965; Norrby, 1969) or the hemagglutinin, neuraminidase, and nucleoprotein antigens of influenza viruses (Schild and Pereira, 1969; Schild, 1970).

It is important to recognize that useful information is obtained only when two antigens are tested against a single serum. When two antisera prepared against cross-reacting antigens are tested against only one of the antigens, a pattern of coalescence of precipitin lines is always obtained (Van Regenmortel, 1966; Scott, 1973). The fusion of precipitin lines, in this case, simply reflects the fact that different antigenic regions on the surface of the virion do not diffuse independently (Grogan *et al.*, 1964). This fact is not always appreciated (Sim and Watson, 1973; Reinganum and Scotti, 1976) and, as a result, the misnomer "reaction of identity," used to describe the merging of lines, often leads authors to the erroneous conclusion that two antigens are serologically identical.

Serological comparisons between related antigens often require that antisera be cross-adsorbed with heterologous antigens. This is most conveniently done by the method of intragel adsorption (Van Regenmortel, 1967). The antigen preparation used for absorption is allowed to diffuse in the gel and will thereby establish a concentration gradient around the well. When, at a later stage, the antiserum is allowed to diffuse from the same well, the cross-reacting antibodies will be fully precipitated in the gel in the zone of equivalence. Unadsorbed antibodies will diffuse freely and will form a residual precipitin line with the homologous antigen (Von Wechmar and Van Regenmortel, 1968; Wetter and Luisoni, 1969; Granett and Shalla, 1970; Bell and Orlob, 1977). This method of serological adsorption has been used to establish that the capsids of certain viruses possess some antigenic determinants that are absent on the corresponding depolymerized capsid subunits (Van Regenmortel, 1978). These new antigenic specificities, called neotopes (Van Regenmortel, 1966), are a reflection of the quaternary structure of viral proteins and could arise either through conformational changes or through the juxtaposition of amino acid residues from neighboring subunits (Norrby and Wadell, 1972; Eppstein and Thoma, 1977).

Large viruses and elongated ones with a length above 500 nm diffuse poorly in 0.5–1.0% agar gel and must be degraded into smaller fragments in order to be studied by immunodiffusion (Purcifull and Gooding, 1970; Shepard, 1972; Esposito *et al*, 1977; Miller and Van der Maaten, 1977; Harrap *et al.*, 1977). Purcifull and Batchelor (1977) have listed 35 plant viruses that have been dissociated with sodium dodecylsulfate in order to permit immunodiffusion studies. Serological relationships between several potyviruses were studied with antisera prepared against their dissociated protein subunits. It was found that the subunits from different viruses were more closely related serologically

than the corresponding intact virions (Shepard *et al.*, 1974). This may be due to the fact that a larger surface area of the native viral subunit is immunochemically expressed in the monomer, compared with the capsid (Van Regenmortel and LeLarge, 1973). Another explanation is that the partial denaturation of the protein reveals internal sequence similarities that are buried in the native molecule (Arnon and Maron, 1971).

5.5. Radial Immunodiffusion

In radial immunodiffusion, one of the reactants, usually the antigen, is allowed to diffuse into a gel containing a uniform concentration of the other reactant. The antigen is deposited in a small well, cut in the gel layer, and diffuses radially into the antibody-containing agar. A ring of precipitation is formed around the charged well and will increase in diameter until such time as the amount of available antigen has been consumed (Mancini *et al.*, 1965). When the diameter of the ring no longer increases, a state reached only after several days, there is a linear relationship between antigen concentration and area of precipitate. In practice, quantitative measurements of antigen concentration are made before the maximum ring diameter is reached. This is done by a comparison with the ring diameters produced by standards of known concentration.

Because of their large size, virions diffuse only slowly in 1% agar gel. As a result, high concentrations of virus are needed to produce rings of precipitation of sufficient diameter to be useful in quantitative measurements. The method has been found to give satisfactory quantitative results with isometric plant viruses of 30 nm diameter (Richter *et al.*, 1976; Juretic and Mamula, 1978), although its ideal range of application lies with smaller antigens such as dissociated viral components.

The radial immunodiffusion procedure can also be reversed, i.e., the intact virions can be incorporated in the gel and the corresponding antiserum deposited in a well. This method was introduced by Schild *et al.*, (1972) to assay influenza virus antibodies and was also used to measure antibodies against adenovirus and vaccinia virus (Grandien and Norrby, 1975; Prakash *et al.*, 1977). In this type of test, the virions are immobilized in the gel as single particles, separated from each other by a distance equal to about 50 times their particle diameter. The ring of "precipitation" in this case is in fact a halo caused by increased light scattering of virions coated with antibody molecules (Schild *et al.*, 1972; Mostow *et al.*, 1975; Norrby *et al.*, 1977). It is also possible to determine

antibody concentrations by incorporating serial dilutions of antiserum in the gel and measuring the precipitation rings produced by standard virus preparations (Cowan and Wagner, 1970; Wagner *et al.*, 1972).

Radial immunodiffusion tests have been used extensively in plant virology to diagnose viral infections of economically important crops (Shepard and Secor, 1969). A mass testing program for potato viruses, based on the use of virions degraded by compounds such as pyrrolidine and pyridine, has been developed (Shepard, 1972).

Simplified techniques have been described which are especially adapted to large-scale screening but which still retain a sensitivity of about 1 μg antigen/ml. Slack and Shepherd (1975) eliminated tissue grinding, well cutting and chemical treatment of the antigen by embedding small pieces of infected leaf tissue in the antibody-containing agar. Van Slogteren (1976) deposited small drops of agar containing the antiserum on the bottom of a petri dish and placed droplets of plant juice containing degraded virus next to them. Precipitation inside the agar drops was observed under a dissecting microscope. Compared to radial diffusion in plates, this method achieved a twelvefold saving of antiserum.

5.6. Radial Hemolysis

Radial hemolysis combines the accuracy of radial immunodiffusion measurements with the sensitivity of hemagglutination inhibition tests. Virus-sensitized erythrocytes are incorporated in an agarose gel, and the corresponding viral antiserum, placed in wells, is allowed to diffuse radially into the gel (Russel *et al.*, 1975). In the presence of complement, a ring of hemolysis is produced around the wells. The technique has been used to assay antibodies to the hemagglutinin (Schild *et al.*, 1975) and neuraminidase antigens (Callow and Beare, 1976) of influenza virus, as well as antibodies to mumps virus (Grillner and Blomberg, 1976).

5.7. Immunoelectrophoretic Techniques

5.7.1. Immunoelectrophoresis

Immunoelectrophoresis was introduced in order to analyze complex mixtures of antigens that are not readily resolved by immunodiffusion. The antigens are first separated by electrophoresis in one dimen-

sion and are then allowed to diffuse in a second dimension toward a trough filled with antiserum (Williams, 1971). The main advantage of the method is that the antigens are defined by two independent criteria: electrophoretic mobility and antigenic specificity. The method provides mainly qualitative information, but has nevertheless been found useful in all areas of virology (Van Regenmortel, 1966; Schmidt and Lennette, 1973; Ball, 1974).

5.7.2. Counterimmunoelectrophoresis

Counterimmunoelectrophoresis is based on the fact that, in agar gel electrophoresis experiments at pH 7–8, most antigens possess a relatively high negative charge and move toward the anode, whereas antibodies that have a lower negative charge are transported toward the cathode by the electro-endosmotic flow. As a result, the two reactants, when placed in opposite wells, will migrate toward each other and will form precipitation lines very rapidly. Furthermore, much smaller quantities of antigen will be detectable than by immunodiffusion. The method, also known as immunoosmophoresis or electrosyneresis, has been applied to the detection of plant viruses (Ragetli and Weintraub, 1964; John, 1965) and Australia antigen (Gocke and Howe, 1970) and to the diagnosis of rubella and rotavirus infections (Grauballe *et al.*, 1975, 1977; Middleton *et al.*, 1976).

5.7.3. Rocket Immunoelectrophoresis

Rocket immunoelectrophoresis is a quantitative method for the measurement of antigen concentration (Laurell, 1966). Antigen samples to be compared are introduced in small wells cut side by side in a layer of antibody-containing agarose gel. When an electric current is applied, the migration of antigen into the gel leads to rocket-shaped precipitation patterns. The heights of these patterns are directly proportional to antigen concentration. The concentration of test samples can be calculated from a standard curve based on known dilutions of a reference preparation (Weeke, 1973*a*). This technique was found to be as sensitive as, but more rapid than, radial immunodiffusion for quantifying the structural proteins of influenza virus (Oxford and Schild, 1977). It has also been found useful for measuring the concentration of the major internal polypeptides of Rauscher murine leukemia virus (Robinson *et al.*, 1977). This protein has a low electrophoretic mobility but could nevertheless be measured by rocket electrophoresis, after its negative

charge had been enhanced by treatment with sodium dodecyl sulfate. In plant virology, the technique has been used to measure the concentration of the whole virions and dissociated subunits of cucumber mosaic virus (Havranek, 1978*a*,*b*).

5.7.4. Crossed Immunoelectrophoresis

Crossed immunoelectrophoresis involves first the electrophoretic separation of a mixture of antigens in agarose gel and then a second electrophoresis, at right angles to the first, into a gel containing antibodies (Weeke, 1973*b*). This method provides a higher resolution than classical immunoelectrophoresis and also allows quantitation of the individual antigenic components. It is particularly suited to the analysis of complex antigen–antibody systems, such as the large number of antigenic components of herpes simplex virus (Vestergaard *et al.*, 1977; Norrild *et al.*, 1977). Precipitates can be cut out from crossed immunoelectrophoresis gels and used to immunize rabbits. Vestergaard (1975) used this method to produce antisera suitable for typing herpes virus isolates.

6. TESTS WITH LABELED ANTIBODIES

6.1. Immunofluorescence

Antibodies labeled with fluorescent compounds such as fluorescein isothiocyanate have been used for many years in the cellular localization of viral antigens (Schmidt and Lennette, 1973). Fluorescent antibodies are able to combine with viral antigens present at the surface of infected cells, and the complex can be observed with an optical microscope using ultraviolet light. The general methodology of immunofluorescent staining has been presented in detail in several reviews (Gardner and McQuillin, 1974; Nairn, 1975; Peters and Coons, 1976; Kawamura, 1977; Faure *et al.*, 1977).

6.1.1. Direct Staining Procedure

In the direct staining procedure, the viral antibody is conjugated to the fluorescent dye and serves directly as a specific stain to detect viral antigens. Although it is not as sensitive as the indirect method, this procedure is less subject to nonspecific background staining. Viral antisera should be shown to be free of antibodies against host anti-

bodies by testing them against uninfected control cells. Unwanted antibodies can be removed by absorption of antisera with suitable preparations of host antigens (Kawamura, 1977; Nairn, 1975; Johansson *et al.*, 1976). The direct staining method is considered more reliable than other fluorescent methods for identifying many different viruses (Emmons and Riggs, 1977).

6.1.2. Indirect Staining Procedure

The indirect staining procedure consists of first allowing unlabeled viral antiserum to react with the virus. After washing the preparation to remove extraneous serum proteins, the presence of bound antibodies is revealed by a second reaction with fluorescent antiglobulin antibodies. These antibodies are prepared against the globulin of the animal species from which viral antibody was obtained. A single fluorescent antibody conjugate will thus be suitable for the detection of a large number of viruses, provided that all the viral antisera used in the primary reaction are prepared in a single animal species. Another advantage of the indirect procedure is that it is more sensitive than the direct one because the intermediate layer of viral antibody appears to increase the surface area available for the binding of the fluorescent label. On the other hand, the indirect method is more likely to produce nonspecific staining and therefore requires very stringent specificity controls (Emmons and Riggs, 1977).

6.1.3. Anticomplement Staining Procedure

The anticomplement staining procedure employs unlabeled viral antibody (IgG) that will fix complement following its interaction with antigen. An anti-C3 fluorescent antibody conjugate is used to detect the presence of bound complexes (Laing, 1974). The sensitivity of this technique is superior to that of the direct staining procedure.

6.1.4. Viral Diagnosis

In addition to its use in classical histological studies of experimental infections, the immunofluorescence procedure has been applied increasingly to the rapid diagnosis of viral infections. It has been especially valuable in the diagnosis of rabies (Lee *et al.*, 1977). Present methods of fluorescence microscopy used in clinical virology make it possible to diagnose many viral infections within a few hours of the

patient's admission to hospital (Emmons and Riggs, 1977; Gardner, 1977).

Comparative studies of the value of immunofluorescence and immunoperoxidase procedures (Kurstak *et al.*, 1977) for identifying viruses in tissue culture have shown that both methods possess similar sensitivity and specificity (Wicker, 1971; Hahon *et al.*, 1975). However, the immunoperoxidase assay is easier to perform and to interpret, and it provides a permanent record of the data (Benjamin, 1974; Benjamin and Ray, 1974; Herrmann *et al.*, 1974).

In plant virology, fluorescent antibodies have been applied successfully in the detection of different virus strains in infected protoplasts (Otsuki and Takebe, 1978) and of citrus tristeza virus in various tissues of infected citrus (Tsuchizaki *et al.*, 1978).

6.2. Immunoenzymatic Tests

Enzyme-labeled antibodies have been successfully applied for some years to the localization of antigens in tissue sections, at both the optical and the electron microscope level (Wicker and Avrameas, 1969; Faure *et al.*, 1977). When it became clear that enzyme immunoassays were also eminently suited for the quantitative determination of antigens and antibodies (Engvall and Perlmann, 1971; Van Weemen and Schuurs, 1971), the method was rapidly adopted by several laboratories for the serodiagnosis of parasitic and viral diseases (Voller *et al.*, 1974, 1976). The method has been successfully applied to the detection of measles, rubella, mumps, and Newcastle disease (Voller and Bidwell, 1977).

Numerous variations of enzyme-linked immunosorbent assays (ELISA) exist, depending on whether the antigen or antibody is being measured. Most investigators use plates of polystyrene or polyvinyl chloride that are commonly employed for hemagglutination and complement fixation tests. The indirect ELISA method used for measuring viral antibody (Voller and Bidwell, 1977) consists of the following steps: (1) coating of wells of a microtiter plate with a standard virus preparation, (2) binding of specific antibody present in a test serum to the virus, (3) binding of an enzyme-labeled antiglobulin serum to the viral antibodies, and (4) revealing the presence of bound enzyme by a colorimetric reaction with a suitable substrate. The initial coating step requires the presence of 0.05 M bicarbonate pH 9.6 buffer. Between each successive incubation with the different reagents, the wells are washed several times with phosphate-buffered saline containing 0.05% Tween. The enzyme most commonly used for preparing the conjugate is

alkaline phosphatase. Its presence in the well is detected by a yellow coloration following the addition of the substrate. In addition to visual observation, the results can be expressed quantitatively by measuring the absorbance at 405 nm. A colorimeter which measures the absorbance directly in the wells has been described (Clem and Yolken, 1978).

For the detection of plant viruses, most workers used the "double antibody sandwich" form of ELISA. In this method, the wells are first coated with antivirus globulin, and the virus in test samples is then trapped by the adsorbed antibody. The presence of trapped virus is revealed by an enzyme-labeled antivirus globulin. This method necessitates the preparation of a different antibody conjugate for each virus to be tested.

When virus antiserum prepared in two animal species is available, it is possible to combine the indirect and "double antibody sandwich" methods. This approach was followed by Scherrer and Bernard (1977) to detect the presence of rotavirus in fecal samples and of rotavirus antibodies in calf sera. The assay involved the following steps: (1) coating of wells with the globulin fraction of a rotavirus antiserum prepared in rabbits, (2) incubation with virus, (3) incubation with antirotavirus bovine antiserum, (4) incubation with purified antibovine antibodies conjugated with alkaline phosphatase, and (5) colorimetric revelation of bound enzyme. The antibodies to bovine IgG used in step 4 were prepared in rabbits and were purified by means of a polymerized bovine IgG immunoadsorbent (Avrameas and Ternynck, 1969). This method made it possible to detect rotavirus at concentrations as low as 20–30 ng/ml. Using a different method that involved antirotavirus globulin coupled to horseradish peroxidase, Ellens and De Leeuw (1977) found that 10^5–10^7 rotavirus particles/ml could be detected by ELISA. These authors concluded that the presence of rotavirus in fecal samples was detected more efficiently by ELISA than by electron microscopy and immunoelectroosmophoresis.

The extreme sensitivity of ELISA approaches that of radioimmunoassays and allows for the detection of as little as 1–10 ng/ml virus (Clark *et al.*, 1976; Kelly *et al.*, 1978; Devergne *et al.*, 1978). This has made it possible, for instance, to detect cucumber mosaic virus in single viruliferous aphids (Gera *et al.*, 1978), and various iridescent viruses in individual larvae of *Galleria mellonella* (Kelly *et al.*, 1978). ELISA has been reported to be about 100 times more sensitive than biological assays currently used for detecting potato virus Y and plum pox virus infections (Clark *et al.*, 1976; Gugerli, 1978). Enzyme immunoassays

are extremely convenient for measuring large numbers of samples and are therefore the method of choice for large-scale epidemiological screening as well as for determining the incidence of virus infection in crops by batch testing. The main limiting factors at the present time reside in the collecting, handling, and preparation of individual extracts (Thresh *et al.*, 1977; Clark and Adams, 1977). These factors limit somewhat the advantages of semiautomated apparatus that allows large numbers of samples to be assayed very rapidly.

Numerous reports indicate that ELISA is capable of discriminating between closely related virus strains. Its specificity is much greater than that of precipitin tests and equals that of neutralization tests (Mills *et al.*, 1978). A single antiserum may fail to detect different serotypes of the same virus, and a pool of antisera specific for various serotypes may be required for the detection of a broad range of strains (Barbara *et al.*, 1978; Kelly *et al.*, 1978; Lister and Rochow, 1979; Rochow and Carmichael, 1979). In some cases, when the homologous conjugated antibody is replaced by a heterologous conjugate, the detection of a cross-reaction between two strains is greatly impeded or even impossible (Koenig, 1978; Barbara *et al.*, 1978). This phenomenon is probably caused by a reduction in antibody avidity following conjugation with the enzyme. According to Koenig (1978), the specificity of the test is so great that some closely related strains (i.e., strains with a difference of two twofold dilution steps separating homologous from heterologous serum titers) cannot be shown to be related by ELISA. The low avidity of the antibody conjugate is also demonstrated by the fact that its binding to the virus is completely inhibited by an equivalent amount of simultaneously added native antibodies. This phenomenon can be put to good use in that it allows for the elimination of unwanted reactions between certain virus antisera and host antigens (Koenig, 1978). Contaminating antihost antibodies, if present in the enzyme-labeled virus antiserum, can be prevented from interfering in the reaction by adding unlabeled antibodies to normal plant proteins.

The extreme strain specificity of the double antibody sandwich method of ELISA could be an advantage when it is important to discriminate between different virus strains, but, more often, it is a drawback in diagnostic work.

When it is necessary to detect various unidentified serotypes of a virus, the indirect form of ELISA which utilizes an anti-globulin enzyme conjugate is much to be preferred. This was demonstrated in a recent comparison of the value of different ELISA procedures for detecting a wide range of tobacco mosaic virus strains (Van Regen-

mortel and Burckard, 1980). By combining the indirect and double antibody sandwich methods, it was possible to detect distantly related serotypes with antisera to one strain only (see Fig. 1). The serological distance between the various tobacco mosaic virus strains used in this study was as great as is likely to be encountered in any group of related serotypes. It is clear, therefore, that the indirect form of ELISA is the method of choice for diagnosis.

6.3. Radioimmunoassay

In radioimmunoassay procedures, either the viral antigen or the antibody is labeled with a radioisotope. The radioactivity bound in the antigen–antibody complex is measured after the unbound labeled reagent has been removed from the complex. Two main procedures can be distinguished. In solid-phase systems, one of the reactants is bound to the surface of some material, and all reactions occur at that site. In liquid phase assays, all the reactants are in a liquid suspension.

The methodology and applications of radioimmunoassays in virology have been summarized by Daugharty and Ziegler (1977). The reader is referred to that review, which covers advances up to 1976. Subsequent work has been concerned mainly with the development of additional solid-phase procedures that meet the requirements of speed, sensitivity, and large-scale operation (Middleton *et al.*, 1977; Yung *et al.*, 1977). Using an indirect radioimmunoassay with ^{125}I-labeled rabbit antibovine IgG for detecting rotavirus antibody, Babiuk *et al.* (1977) found that the assay was a thousandfold more sensitive than the counterimmunoelectrophoresis technique.

The sensitivity of direct and indirect solid-phase radioimmunoassays for detecting polyhedron protein of an insect baculovirus have been compared (Crawford *et al.*, 1977). The direct assay using ^{125}I-labeled rabbit immunoglobulin could detect 200 ng of polyhedron protein, whereas the indirect assay, using ^{125}I-labeled sheep antirabbit immunoglobulin, could detect 50 ng of polyhedron protein. Additional assays have been described for detecting viral antibody and antigen in herpes simplex and hepatitis A infections (Forghani *et al.*, 1975; Enlander *et al.*, 1976; Purcell *et al.*, 1976). A direct solid-phase radioimmunoassay, which involves binding of purified radiolabeled IgG to influenza virus hemagglutinins, has been used to study the molecular arrangement of antigenic determinants on the hemagglutinin molecule (Russell and Jackson, 1978).

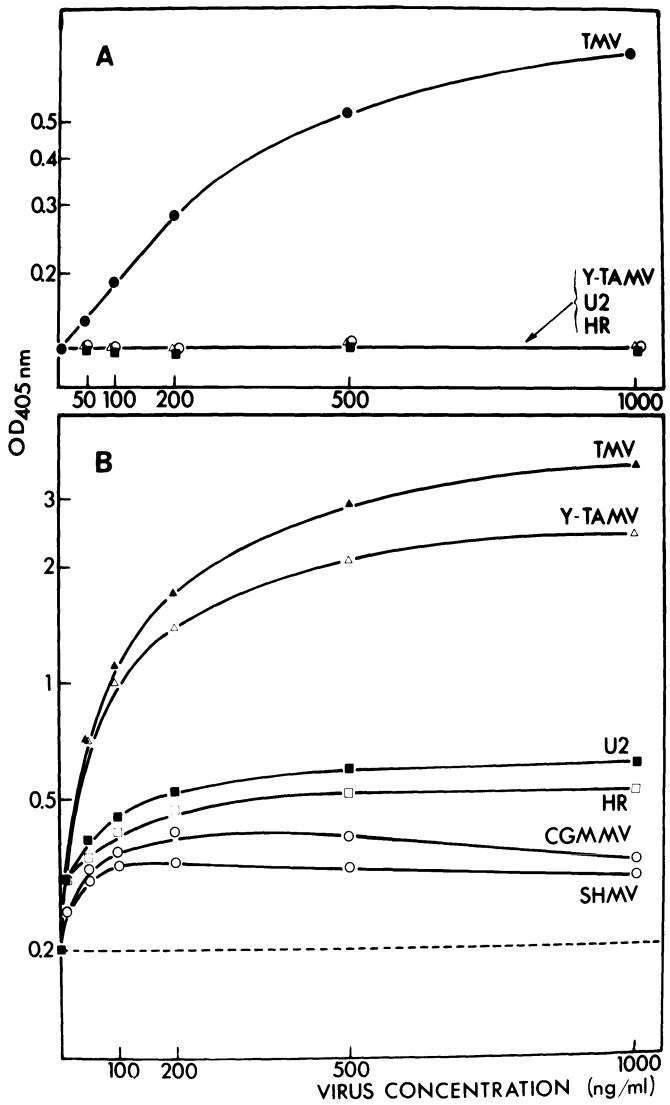


Fig. 1. Comparison of double antibody sandwich method (A) and indirect method (B) of ELISA for detecting serologically distinct strains of tobacco mosaic virus. The serological differentiation indices (SDI) between TMV and strains Y-TAMV, HR, U2, SHMV, and CGMMV are 1.2, 2.1, 2.7, 5.3, and 7.0 (Van Regenmortel, 1975). These values represent the difference between homologous and heterologous precipitin titers expressed as Neg Log_2. *Method A:* Coating of wells was done with goat anti-TMV globulin; the different viruses were incubated for 3 hr; the rabbit anti-TMV alkaline phosphatase conjugate was incubated for 2 hr. *Method B:* Same coating as in method A; after 3-hr adsorption of the viruses, rabbit anti-TMV globulin was allowed to interact for 2 hr. The enzyme conjugate (goat anti-rabbit globulin labeled with alkaline phosphatase) was incubated for 2 hr (Van Regenmortel and Burckard, 1980).

7. COMPLEMENT FIXATION

Complement fixation is extensively used in the diagnosis of a large number of virus infections of man and animals. It is based on the fact that an enzymatic system of serum proteins, known as complement, binds to antibody molecules when these have reacted with the corresponding antigen. If the antigen is associated with an erythrocyte cell surface, the fixation and concomitant activation of complement by the bound antibody leads to lysis of the red blood cell.

The complement fixation test is a two-stage procedure, involving the test system under study and an indicator system consisting of sheep erythrocytes and their corresponding antibodies. The complement present in both the test antiserum and the anti-sheep-erythrocyte serum (hemolysin) is destroyed by heating. A small amount of guinea pig complement sufficient to cause complete hemolysis of the erythrocytes present in the indicator system is added to the test antiserum.

The first part of the test consists in an overnight incubation of antigen, antibody, and complement. Depending on the amount of antigen and antibody present, different amounts of complement will be fixed. In the second part of the reaction, the sheep erythrocytes and their antibodies are introduced as an indicator. Any unused complement left in the test system will bind to the red blood cells; this will cause a certain amount of lysis. The amount of hemoglobin released from the lysed cells can be measured photometrically and is inversely proportional to the extent to which antigen and antibody have interacted and fixed complement in the test system.

For maximum sensitivity, it is important to determine, by a preliminary titration, the minimum amount of hemolysin and complement needed to lyse a predetermined number of red blood cells. The preparation of the reagents and the various titration procedures have been described in detail (Kabat and Mayer, 1961; Bradstreet and Taylor, 1962; Grist *et al.*, 1974; Mayr *et al.*, 1977).

The main difficulty encountered with this technique is the so-called anticomplementary effect. This refers to the ability of certain sera and antigen preparations to fix complement in the absence of any antigen–antibody reaction. When cell extracts or tissue culture exudates show anticomplementary activity, it is necessary to further purify the antigen. The anticomplementary activity of antisera can often be overcome by utilizing a sufficiently high serum dilution in the test. The anticomplementary activity may also be removed by a preliminary incubation with guinea pig complement, followed by heating at 60° C (Mayr *et al.*, 1977).

7.1. Microplate Technique

The microplate technique is based on the 100% hemolysis end point determined by visual inspection of the wells (Bradstreet and Taylor, 1962). The reaction can also be done in wells of cell culture trays in which virus-infected cells have been incubated. This method was used to titrate lymphocytic choriomeningitis virus in cell cultures (Gschwender and Lehmann-Grube, 1975). The microplate technique is widely used for serological diagnosis (Schmidt and Lennette, 1973) and is also suitable for strain differentiation (Forman, 1974). However, small antigenic differences between virus strains are more easily detected in tube tests, in which the degree of hemolysis is measured photometrically.

7.2. Quantitative Microcomplement Fixation Test

Various modifications of quantitative complement fixation tests in tubes have been described. In all these tests, the percentage of complement fixation is calculated from photometric measurements of the degree of hemolysis. For a given antiserum dilution, a plot of the percentage of complement fixed by increasing quantities of antigen takes the form of a bell-shaped curve (Levine and Van Vunakis, 1967). This test has been extensively used for the study of antigenic relationships (Scott, 1965; Prager and Wilson, 1971). Such studies are often done by means of inhibition of complement fixation tests, in which various quantities of inhibitor are incubated with antiserum. The residual amount of complement fixation is then measured, using the concentration of antigen which in the absence of inhibitor corresponded to the maximum of the bell-shaped curve. This method has been used, for instance, for studying the antigenic determinants of tobacco mosaic virus protein (Benjamini *et al.*, 1964). By inhibiting the complement fixation reaction by means of tryptic peptides obtained from the coat protein, it was possible to locate several antigenic regions in the capsid protein of this virus (Milton and Van Regenmortel, 1979).

8. IMMUNOELECTRON MICROSCOPY

The visualization of immunological reactions in the electron microscope is one of the most sensitive methods of antigenic analysis. Two different approaches can be distinguished, depending on whether the antigen is in suspension or is present in a cell or tissue. Histological

methods of immunocytology will not be reviewed in detail here (see Wagner, 1973; Hämmerling, 1976). These methods entail the use of labeled antibodies as a "stain," the most common labels being ferritin (Rifkind, 1976; Oshiro *et al.*, 1977) and enzymes such as horseradish peroxidase and alkaline phosphatase (Kraehenbuhl and Jamieson, 1976). Enzyme-labeled antibodies can be used for preembedding staining, in which case they are allowed to diffuse inside fixed cells and interact with antigenic sites prior to thin sectioning. Another procedure consists of applying the labeled antibody after embedding and thin sectioning of the tissue, a method which reveals only antigens that are exposed at the surface of the section. The applications of immunocytological methods for the localization of viral antigens in infected cells have been reviewed by Howe *et al.* (1969), Kurstak and Morisset (1974), and Kurstak *et al.* (1977). The methods have been described in detail by Faure *et al.* (1977) and Kurstak *et al.* (1977).

The following discussion of immunoelectron microscopy will be limited to cases where the virions are in suspension and the antigen–antibody complex is visualized directly on the electron microscope grid. A positive serological reaction can be recognized in the electron microscope by three different phenomena that may occur together or separately, i.e., clumping, decoration, and trapping on antibody-coated grids.

8.1. Clumping

The clumping method consists of mixing a suspension containing virus particles with a suitable dilution of an antiserum and incubating the mixture to allow complexes to form. The complexes can be centrifuged to a pellet in order to increase the sensitivity of detection, or they can simply be deposited on a microscope grid. An agar surface can be used to absorb impurities (Kelen *et al.* 1971). Negative staining is generally used, and aggregates can be visualized with as little as 10^6 virions present in a suspension (Almeida and Waterson, 1969). The clumping procedure is especially valuable when the virus concentration is too low to see the particles directly or when virions with a similar morphology have to be differentiated (Fauvel *et al.*, 1977).

When sufficient antibody is present in the mixture, the virions will be covered by a more or less continuous layer of antibody molecules, an effect known as decoration (Milne and Luisoni, 1977). The halo which then surrounds the virions leads to a fuzzy outline of the particles which is very characteristic (Almeida and Waterson, 1969). An excess of anti-

body may inhibit clumping in the same way as it inhibits the classical precipitation reaction.

The clumping method is extremely useful for identifying elusive human viruses that cannot be cultivated or that do not normally grow to sufficiently high titer to be identified by classical techniques, e.g., the wart viruses, coronaviruses, rhinoviruses, and the infectious hepatitis virus (Kapikian *et al.*, 1972*a*; Feinstone *et al.*, 1973; Doane and Anderson, 1977). Other uses of the method include the identification of plant viruses by the leaf-dip procedure (Ball, 1971; Langenberg, 1974), the demonstration of distinct antigens on the different morphological forms of hepatitis B particles (Neurath *et al.*, 1976; Stannard and Moodie, 1976), the establishment of antigenic relationships between certain papovaviruses (Penney and Narayan, 1973), and the localization of antigenic determinants on the tip of isolated hemagglutinin molecules of influenza virus (Wrigley *et al.*, 1977).

As in other immunoelectron microscopic procedures, the diagnostic reagent can simply be convalescent serum from patients, as in the original study of viral gastroenteritis (Kapikian *et al.*, 1972*b*). Purified globulins have also been used, as well as heat-inactivated antisera, to avoid complications due to the presence of complement (Almeida and Waterson, 1969). It is also possible to make the method more sensitive by using antiglobulin serum to further aggregate the virus–antibody complexes (Edwards *et al.*, 1975).

8.2. Decoration

The phenomenon of decoration can be visualized independently of the clumping reaction, provided that the virions are first immobilized by adsorption onto the microscope grids. When an excess of antibody is used, prior adsorption of virions to grids may be unnecessary since the particles are often completely covered with antibody molecules. In the case of elongated plant viruses that are difficult to purify, an advantage of the decoration method is that any problems resulting from contamination with antibodies to host antigens are reduced, since there is direct evidence that the virus is reacting.

The decoration method is especially valuable for the study of virions that possess a complex morphology, since it allows the site of antibody attachment to be located (Luisoni *et al.*, 1975). Seven different antigenic components on the surface of phage T4 have been distinguished in this way by means of specific antibody (Yanagida and Ahmad-Zadeh, 1970). The antibody fractions used in this work were

obtained by adsorbing anti-T4 wild-type serum with lysates of phage mutants defective in a specific gene product. After absorption, the antiserum contained only antibodies directed to the missing gene product. These antibodies were found by electron microscopy to bind to distinct areas of the phage particle which correspond to the different gene products. A similar approach was employed to locate different antigens on the surface of phage $\phi 29$ (Tosi and Anderson, 1973). In another study, polyheads of T4 phage made up of uncleaved phage head protein were shown by immunoelectron microscopy to be antigenically different from phage capsids (Yanagida, 1972).

8.3. Trapping

The trapping of plant viruses to electron microscope grids coated with specific antiserum was first described by Derrick (1973), who called the technique "serologically specific electron microscopy." The term "trapping" is used here to avoid confusion with other microscopical procedures that also rely on the visualization of virus–antibody interaction.

The method consists of the following steps. Electron microscope grids with parlodion–carbon films are allowed to float on drops of dilute virus antiserum for about 30 min. During this time, a layer of serum proteins is adsorbed to the film; excess protein is removed by washing with a buffer solution. The coated grids are then placed for 1–2 hr on drops of virus suspensions or extracts of infected tissue. Antibody molecules adsorbed to the grid specifically trap homologous virus particles. Salts and contaminants are removed by washing, and virions can be visualized after metal shadowing or negative staining.

The trapping method has been applied to the identification and quantitative analysis of numerous elongated and isometric plant viruses (Derrick and Brlansky, 1976; Paliwal, 1977; Beier and Shepherd, 1978; Milne and Lesemann, 1978; Nicolaïeff and Van Regenmortel, 1980) as well as for detecting double-stranded RNA in extracts of tobacco infected with tobacco mosaic virus (Derrick, 1978). Although mainly used by plant virologists, the trapping method will no doubt find many applications in other areas of virology as well. Recently, for instance, the method was found useful for detecting human rotaviruses in crude fecal extracts (Nicolaïeff *et al.*, 1980). Compared to standard microscopy which could detect rotavirus particles in only 20% of the specimens, the trapping procedure revealed virus particles in 71% of them.

For the quantitative assay of plant viruses in crude extracts, trapping on antiserum-coated grids is as sensitive as local lesion assays (Beier and Shepherd, 1978). Several authors have found that the log of the number of virions trapped decreases linearly with dilution and that as little as 10 ng/ml of virus can be detected (Derrick, 1973; Paliwal, 1977; Beier and Shepherd, 1978).

Optimal conditions for the test have to be determined in each case. The binding of virions to antibody-coated grids is inhibited when the antiserum used for coating is not sufficiently diluted. A similar effect is probably responsible for the fact that grids coated with normal serum adsorb practically no virions, whereas uncoated grids often adsorb a considerable number of particles. However, the number of normal serum proteins needed to prevent the particles of different viruses from adsorbing to grids is highly variable (Nicolaïeff and Van Regenmortel, 1980). This means that it is necessary to use, as controls, grids coated with normal serum diluted to the same extent as the specific antiserum.

Other parameters which may influence the extent of trapping are the temperature (Paliwal, 1977), the presence of NaCl and sucrose in the buffer (Derrick and Brlandsky, 1976; Beier and Shepherd, 1978), and the incubation time. The sensitivity of the technique can also be increased by precoating the grids with protein A from *Staphylococcus aureus* prior to coating them with specific antiserum (Shukla and Gough, 1979; Nicolaïeff *et al.*, 1980). Furthermore, it appears that maximum sensitivity and specificity can be obtained when the trapping and decoration effects are combined (Milne and Luisoni, 1977). It seems likely that both procedures will be used increasingly in the future since they offer many advantages compared to the simple clumping method.

The strain specificity of the serological trapping technique has been measured with a series of tobacco mosaic virus strains (Nicolaïeff and Van Regenmortel, 1980). Since the extent of serological cross-reactivity between these strains is known with a considerable degree of precision (Van Regenmortel, 1975), it was possible to determine how closely related serologically two strains have to be in order to be trapped on grids coated with heterologous antiserum. It was found that when two strains differed by a serological differentiation index (SDI) of about 3 (i.e., when the number of twofold dilution steps separating homologous and heterologous precipitin titers was about 3), the strains could still be detected with a single heterologous antiserum. However, when the SDI between two strains was as large as 4, the serological cross-reactivity was too low to obtain sufficient trapping on grids coated with heterologous antiserum.

9. APPLICATIONS OF SEROLOGICAL METHODS

In the following section, the different uses of serological techniques in virology will be briefly summarized.

9.1. Diagnosis

The diagnosis of viral infections represents the most widely used application of serological investigations in all fields of virology. Two main approaches can be distinguished. Virus material isolated from infected tissue may be tested for its ability to react with any one of a series of reference antisera prepared against different viruses. This is the only approach that can be used in plant virology, and its success in large-scale serological screening is well documented (Wetter, 1965; Shepard, 1972; Hollings, 1975).

In the case of viruses which infect man and vertebrates, it is also possible to diagnose an infection by demonstrating the appearance or increase of viral antibody in the blood stream of the infected host. This approach is amply illustrated in a number of extensive reviews of diagnostic procedures (Schmidt and Lennette, 1973; Bricout *et al.*, 1974; Kurstak and Kurstak, 1977; Mayr *et al.*, 1977; Hsiung, 1977).

9.2. Evaluation of Vaccines

Neutralization tests are the logical choice for evaluating the protective capacity of an antibody response induced by vaccination, since they directly measure protection against virus challenge. However, such tests are time-consuming and not very convenient for large-scale use. Other methods, such as hemagglutination inhibition, complement fixation, radial hemolysis, and mixed hemadsorption, have also been used successfully for assaying the antibody response produced by vaccination (Cox *et al.*, 1977; Grandien, 1977; Aymard, 1977).

Recently, there has been much interest in the use of subunit vaccines. Vaccination with viral structural components free of genetic material has been advocated on the grounds, first, that it avoids the injection of potentially oncogenic material (Neurath and Rubin, 1971; Rapp and Reed, 1976) and second, because it reduces the pyrogenic activity and toxic side effects of some whole-virus vaccines. Influenza subunit vaccines have been in commercial use for a number of years (Rubin and Tint, 1975). Because of the presence of toxic compounds in

rabies vaccines prepared from infected brain tissue, much effort has been spent in obtaining purified virus dissociation products that have retained their antigenicity. The spike glycoprotein of rabies virus has been purified and shown to possess an excellent immunizing capacity (Wiktor *et al.*, 1973; Cox *et al.*, 1977). With adenovirus and herpesvirus, the protective effect of nucleic-acid-free vaccines has been demonstrated in mice (Mautner and Willcox, 1974; Parks and Rapp, 1975; Kitces *et al.*, 1977).

The immunogenicity of subunit vaccines is generally much lower than that of whole-virus vaccines, and it is in this area that progress is badly needed. Several reports indicate that the efficacy of vaccines can be improved considerably by means of adjuvants (Branche and Renoux, 1972; Kreuter and Speiser, 1976; Langbeheim *et al.*, 1978).

In the case of Semlike Forest virus, protein aggregates in the form of micelles were more immunogenic than the monomeric form of the spike glycoprotein (Morein *et al.*, 1978). It is not clear whether the higher immunogenicity of aggregates is linked to the fact that multivalent antigens with repeating subunits are often thymus independent (Feldman and Basten, 1971; Ströbel, 1974) or whether it reflects the existence of new antigenic determinants induced by the quaternary structure of the proteins. The formation of such neotopes through association of monomers (Van Regenmortel, 1966, 1978) is also likely to occur in the case of glycoproteins and lipid-bound proteins (Neurath and Rubin, 1971). If neotopes contribute significantly to the overall antigenic structure of the virion, antibodies formed as a result of immunization with monomers may fail to neutralize the virus efficiently.

It should also be pointed out that, in many cases, the mechanism of immune protection is not well understood. In several virus systems, it is known that both humoral and cell-mediated immunity play a role (Burns and Allison, 1975; Cappel, 1976), while in others, there is evidence that local secretory antibody production is important (Shvartsman and Zykov, 1976).

9.3. Studies of Virus Structure

Specific antibodies are a powerful tool for localizing different antigenic structures in virions. The position of a specific gene product in the assembled virion can be visualized directly in the electron microscope by the antibody decoration technique (Yanagida and Ahmad-Zadeh, 1970), or it can be inferred from the results of hemagglutination or neu-

tralization tests (Wadell, 1972). Precipitation techniques have been extensively used for studying the various subunits found in influenza and adenoviruses (Norrby, 1969; Schild, 1970). The phenomenon of antigenic variation in influenza viruses has been shown to be related to changes in the primary structure of the hemagglutinin polypeptide (Webster and Laver, 1975). In the case of TMV strains, the degree of serological cross-reaction was found to be correlated with the extent of sequence homology in the coat proteins of the different strains (Van Regenmortel, 1975).

In recent studies, fragments of viral polypeptides obtained by proteolytic and chemical cleavage have been used to determine the precise location of antigenic sites in viral subunits. A glycopeptide which contains a major antigenic determinant of hepatitis B surface antigen particles has been isolated (Burrell *et al.*, 1976; Neurath *et al.*, 1978). Short peptides corresponding to the antigenic determinants of influenza virus hemagglutinin (Jackson *et al.*, 1978) and TMV coat protein have also been studied (Milton and Van Regenmortel, 1979).

9.4. Studies of Viral Replication

The immunoprecipitation techniques described in Section 5.3 have been extensively used for analyzing the synthesis of viral polypeptides in infected cells (Shanmugam *et al.*, 1972). In pulse-chase experiments, the radiolabeled proteins are incubated with viral antibodies and a second antiglobulin serum, and the resulting precipitate is analyzed by gel electrophoresis. In the case of retroviruses, this type of study has made it possible to identify several precursor polypeptides which appear to be processed by host enzymes (Vogt and Eisenman, 1973; Van Zaane *et al.*, 1975; Naso *et al.*, 1975).

9.5. Virus Classification

Serological cross-reactions are one of the most reliable criteria for deciding on relatedness between viruses. Indeed, viruses that are serologically related always share morphological and biochemical characteristics that place them in the same taxonomical group. Sometimes, a particular serological technique is not sufficiently sensitive to show up the presence of common antigens between two viruses. In such cases, cross-immunization with a related virus may be needed for the

heterologous antibody response to be detected (Schmidt *et al.*, 1969; Martin *et al.*, 1972).

In addition to its value for deciding on membership of a group, serology obviously also allows finer distinctions to be made. These can be based on criteria such as (1) cross-neutralization expressed as a ratio of homologous and heterologous neutralization rate constants (Honess and Watson, 1977), (2) the number of common precipitin bands observed when viruses are compared in reciprocal double diffusion tests (Honess and Watson, 1977), and (3) the ratio of homologous to heterologous titers in quantitative precipitin tests (Van Regenmortel, 1975).

Whenever a sufficient number of serologically related members of a group are examined, it is found that they can be arranged in a continuous series of increasingly distantly related entities (Koenig, 1976; Van Regenmortel, 1978). It reminds us that distinctions between close and distant serological relationships are essentially arbitrary, and that serological categories, like all categories, are constructed by the human mind and exist only by virtue of definition (Lwoff and Tournier, 1971).

10. REFERENCES

Abu Salih, H. S., Murant, A. F., and Daft, M. J., 1968*a*, Comparison of the passive haemagglutination and bentonite flocculation tests for serological work with plant viruses, *J. Gen. Virol.* **2**:155.

Abu Salih, H. S., Murant, A. F., and Daft, M. J., 1968*b*, The use of antibody-sensitized latex particles to detect plant viruses, *J. Gen. Virol.* **3**:299.

Adams, M. H., 1959, *Bacteriophages*, Interscience, New York.

Almeida, J. D., and Waterson, A. P., 1969, The morphology of virus-antibody interaction, *Adv. Virus Res.* **15**:307.

Altemeyer, W. A., Mundon, F. K., Top, F. H., and Russel, P. K., 1970, Method for extracting viral hemagglutination-inhibiting antibodies from the nonspecific inhibitors of serum, *Appl. Microbiol.* **19**:785.

Anderer, F. A., and Ströbel, G., 1972*a*, Recognition of conjugated and native peptide determinants. I. Conformational and sequential specificities of rabbit antibodies versus tobacco mosaic virus, *Eur. J. Immunol.* **2**:274.

Anderer, F. A., and Ströbel, G., 1972*b*, Recognition of conjugated and native peptide determinants. II. Enhancement of antibodies with sequential specificity in anti-TMV sera by preimmunization with conjugated peptide antigens, *Eur. J. Immunol.* **2**:278.

Appleyard, G., and Zwartouw, H. T., 1978, Preparation of antigens from animal viruses, in: *Handbook of Experimental Immunology* (D. M. Weir, ed.), Chap. 3, Blackwell, Oxford.

Arnon, R., and Maron, E., 1971, An immunological approach to the structural relationship between hen egg-white lysozyme and bovine α-lactalbumin, *J. Mol. Biol.* **60**:225.

Arquilla, E. R., 1977, Antigen-red cell conjugates prepared with bisdiazobenzidine, in: *Methods in Immunology and Immunochemistry*, Vol. 4, p. 41, Academic Press, New York.

Ashe, W. K., and Scherp, H. W., 1963, Antigenic analysis of herpes simplex virus by neutralization kinetics, *J. Immunol.* **91**:658.

Ashe, W. K., Mage, M., and Notkins, A. L., 1969, Kinetics of neutralization of sensitized herpes simplex virus with antibody fragments, *Virology* **37**:290.

Ashe, W. K., Daniels, C. A., Scott, G. S., and Notkins, A. L., 1971, Interaction of rheumatoid factor with infectious herpes simplex virus-antibody complexes, *Science* **172**:176.

Austin, R. M., and Daniels, C. A., 1974, Interaction of staphylococcal protein A with virus-IgG complexes, *J. Immunol.* **113**:1568.

Avrameas, S., and Ternynck, T., 1969, The cross-linking of proteins with glutaraldehyde and its use for the preparation of immunoadsorbents, *Immunochemistry* **6**:53.

Avrameas, S., Brailovsky, C., and Ternynck, T., 1969, Isolation of viruses using specific immunoadsorbents, *Virology* **37**:508.

Aymard, M., 1977, Comparison of immunological methods in the diagnosis of influenza, *Bull. Inst. Pasteur Paris* **75**:309.

Babiuk, L. A., Acres, S. D., and Rouse, B. T., 1977, Solid-phase radioimmunoassay for detecting bovine (neonatal calf diarrhea) rotavirus antibody, *J. Clin. Microbiol.* **6**:10.

Ball, E. M., 1971, Leaf-dip serology, in: *Methods in Virology*, Vol. V (K. Maramorosch and H. Koprowski, eds.), pp. 445–450, Academic Press, New York.

Ball, E. M., 1974, Serological tests for the identification of plant viruses, *Am. Phytopathol. Soc.*, St. Paul, Minnesota.

Barbara, D. J., Clark, M. F., and Thresh, J. M., 1978, Rapid detection and serotyping of *Prunus* necrotic ringspot virus in perennial crops by enzyme-linked immunosorbent assay, *Ann. Appl. Biol.* **90**:395.

Becht, G., 1968, Properties of erythrocytes stabilized with sulfosalicyl acid and their use in an indirect hemagglutination test with influenza virus RNP antigen, *J. Immunol.* **101**:18.

Becht, H., and Malole, B., 1975, Comparative evaluation of different fixation procedures and different coupling reagents for the demonstration of influenza virus-specific antibodies by the indirect hemagglutination test, *Med. Microbiol. Immunol.* **162**:43.

Beier, H., and Shepherd, R. J., 1978, Serologically specific electron microscopy in the quantitative measurement of two isometric viruses, *Phytopathology* **68**:533.

Bell, C. D., and Orlob, G. B., 1977, Serological studies on virions and polyhedron protein of a nuclear polyhedrosis virus of the cabbage looper, Trichoplusia ni, *Virology* **78**:162.

Benjamin, D. R., 1974, Rapid typing of herpes simplex virus strains using the indirect immunoperoxidase method, *Appl. Microbiol.* **28**:568.

Benjamin, D. R., and Ray, C. G., 1974, Use of immunoperoxidase for the rapid identification of human myxoviruses and paramyxoviruses in tissue culture, *Appl. Microbiol.* **28**:47.

Benjamini, E., Young, J. D., Shimizu, M., and Leung, C. Y., 1964, Immunochemical studies on the tobacco mosaic virus protein. I. The immunological relationship of the tryptic peptides of tobacco mosaic virus protein to the whole protein, *Biochemistry* **3**:1115.

Bercks, R., 1967, Methodische Unterzuchungen über den serologischen Nachweis pflanzenpathogener Viren mit dem Bentonit-Flockungstest, dem Latex-Test und dem Bariumsulfat-Test, *Phytopathol. Z.* **58**:1.

Bercks, R., and Querfurth, G., 1969, Weitere methodische Untersuchungen über den Latextest zum serologischen Nachweis pflanzenpathogener Viren, *Phytophathol. Z.* **65**:243.

Bercks, R., and Querfurth, G., 1971, The use of the latex test for the detection of distant serological relationships among plant viruses, *J. Gen. Virol.* **12**:25.

Bercks, R., Koenig, R., and Querfurth, G., 1972, Plant virus serology, in: *Principles and Techniques in Plant Virology* (C. I. Kado and H. O. Agrawal, eds.), pp. 466–490, Van Nostrand Reinhold, New York.

Berget, P. B., and King, J., 1978, Antigenic gene products of bacteriophage T4 baseplates, *Virology* **86**:312.

Bernstein, M. T., and Stewart, J. A., 1971, Method for typing antisera to herpesvirus hominis by indirect hemagglutination inhibition, *Appl. Microbiol.* **21**:680.

Berry, D. M., and Almeida, J., 1968, The morphological and biological effects of various antisera on avian infectious bronchitis virus, *J. Gen. Virol.* **3**:97.

Bingham, R. W., Madge, M. H., and Tyrrell, D. A. J., 1975, Haemagglutination by avian infectious bronchitis virus—A coronavirus, *J. Gen. Virol.* **28**:381.

Birkbeck, T. H., and Stephen, J., 1970, Specific removal of host-cell or vaccinis-virus antigens from extracts of infected cells by polyvalent disulphide-linked immunosorbents, *J. Gen. Virol.* **8**:133.

Blank, S. E., Leslie, G. A., and Clem, L. W., 1972, Antibody affinity and valence in viral neutralization, *J. Immunol.* **108**:665.

Bradstreet, C. M. P., and Taylor, C. E. D., 1962, Technique of complement fixation test applicable to the diagnosis of virus disease, *Mth. Bull. Minist. Hlth. Lab. Serv.* **21**:96.

Branch, R., and Renoux, G., 1972, Stimulation of rabies vaccine in mice by low doses of polyadenylic: Polyuridylic complex, *Infect. Immun.* **6**:324.

Bricout, F., Joubert, L., and Huraux, J. M., 1974, *Diagnostic Séro-Immunologique des Viroses Humaines et Animales*, Maloine, Paris.

Brown, F., and Cartwright, B., 1960, Purification of the virus of foot-and-mouth disease by fluorocarbon treatment and its dissociation from neutralizing antibody, *J. Immunol.* **85**:309.

Brown, F., and Smale, C. J., 1970, Demonstration of three specific sites on the surface of foot-and-mouth disease virus by antibody complexing, *J. Gen. Virol.* **7**:115.

Brown, F., Talbot, P., and Burrows, R., 1973, Antigenic differences between isolates of swine vesicular disease virus and their relationship to coxsackie B5, *Nature (London)* **245**:315.

Brugh, M., Jr., 1977, Reactivation of Newcastle disease virus neutralized by antibody, *J. Clin. Microbiol.* **5**:410.

Burns, W. H., and Allison, A. C., 1975, Virus infections and the immune responses they elicit, in: *The Antigens*, Vol. III (M. Sela, ed.), pp. 479–574, Academic Press, New York.

Burrell, C. J., Leadbetter, G., Mackay, P., and Marmion, B. P., 1976, Tryptic cleavage

of antibody binding sites from hepatitis B surface antigen particles, *J. Gen. Virol.* **33**:41.

Callow, K. A., and Beare, A. S., 1976, Measurement of antibody to influenza virus neuraminidase by single radial hemolysis in agarose gels, *Infect. Immun.* **13**:1.

Cappel, R., 1976, Comparison of the humoral and cellular immune responses after immunization with live, UV inactivated herpes simplex virus and a subunit vaccine and efficacy of these immunizations, *Arch. Virol.* **52**:29.

Carroll, R. B., Goldfine, S. M., and Melero, J. A., 1978, Antiserum to polyacrylamide gel-purified simian virus 40 T antigen, *Virology* **87**:194.

Carter, J. B., 1973, Detection and assay of *Tipula* iridescent virus by the latex agglutination test, *J. Gen. Virol.* **21**:181.

Cartwright, B., Smale, C. J., and Brown, F., 1970, Dissection of vesicular stomatitis virus into the infective ribonucleoprotein and immunizing components, *J. Gen. Virol.* **7**:19.

Casals, J., 1967, Immunological techniques for animal viruses, in: *Methods in Virology*, Vol. III (K. Maramorosch and H. Koprowski, eds.), pp. 113–198, Academic Press, New York.

Chase, M. W., 1967, Immunization Procedures. I. Immunization of mammals other than man, in: *Methods in Immunology and Immunochemistry*, Vol. I (C. A. Williams and M. W. Chase, eds.), pp. 209–224, Academic Press, New York.

Choppin, P. W., and Compans, R. W., 1970, Phenotypic mixing of envelope proteins of the parainfluenza virus SV_5 and vesicular stomatitis virus, *J. Virol.* **5**:609.

Clark, M. F., and Adams, A. N., 1977, Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses, *J. Gen. Virol.* **34**:475.

Clark, M. F., Adams, A. N., and Barbara, D. J., 1976, The detection of plant viruses by enzyme-linked immunosorbent assay (ELISA), *Acta Hortic.* **67**:43.

Clem, T. R., and Yolken, R. H., 1978, Practical colorimeter for direct measurement of microplates in enzyme immunoassay systems, *J. Clin. Microbiol.* **7**:55.

Conley, M. P., and Wood, W. B., 1975, Bacteriophage T4 whiskers. A rudimentary environmental-sensing device, *Proc. Natl. Acad. Sci. USA* **72**:3701.

Cooney, M. K., Kenny, G. E., Tam, R., and Fox, J. P., 1973, Cross relationships amoung 37 rhinoviruses demonstrated by virus neutralization with potent monotypic rabbit antisera, *Infect. Immun.* **7**:335.

Cooper, N. R., 1979, Humoral immunity to viruses, in: *Comprehensive Virology*, Vol. 15 (H. Fraenkel-Conrat and R. Wagner, eds.), pp. 123–170, Plenum Press, New York.

Corbett, M. K., 1967, Some distinguishing characteristics of the orchid strain of tobacco mosaic virus, *Phytopathology* **57**:164.

Cowan, K. M., 1966, Effect of merthiolate on agar gel diffusion precipitin reactions with foot-and-mouth disease virus, *J. Immunol.* **97**:647.

Cowan, K. M., 1973, Antibody response to viral antigens, *Adv. Immunol.* **17**:195.

Cowan, K. M., and Wagner, G. G., 1970, Immunochemical studies of foot-and-mouth disease. VIII. Detection and quantitation of antibodies by radial immunodiffusion, *J. Immunol.* **105**:557.

Cox, J. H., Dietzschold, B., and Schneider, L. G., 1977, Rabies virus glycoprotein. II. Biological and serological characterization, *Infect. Immun.* **16**:754.

Crawford, A. M., Kalmakoff, J., and Longworth, J. F., 1977, A solid-phase radioimmunoassay for polyhedron protein from baculoviruses, *Intervirology* **8**:117.

Crowle, A. J., 1973, *Immunodiffusion*, 2nd ed., Academic Press, New York.

Cunningham, J. C., Tinsley, T. W., and Walker, J. M., 1966, Haemagglutination with plant and insect viruses, *J. Gen. Microbiol.* **42**:397.

Daniels, C. A., 1975, Mechanisms of viral neutralization, in: *Viral Immunology and Immunopathology* (A. L. Notkins, ed.), p. 79, Academic Press, New York.

Daniels, C. A., Mergenhagen, S. E., and Notkins, A. L., 1970, Measurement of antibody against human IgG by the neutralization of IgG-sensitized herpes simplex virus, *J. Immunol.* **104**:470.

Daugharty, H., and Ziegler, D. W., 1977, Radioimmunoassay in viral diagnosis, in: *Comparative Diagnosis of Viral Diseases*, Vol. II (E. Kurstak and C. Kurstak, eds.) pp. 459–487, Academic Press, New York.

Della-Porta, A. J., and Westaway, E. G., 1972, Rapid preparation of hemagglutinins of togaviruses from infected cell culture fluids, *Appl. Microbiol.* **23**:158.

Della-Porta, A. J., and Westaway, E. G., 1978, A multi-hit model for the neutralization of animal viruses, *J. Gen. Virol.* **38**:1.

Derrick, K. S., 1973, Detection and identification of plant viruses by serologically specific electron microscopy, *Phytopathology* **63**:441.

Derrick, K. S., 1978, Double-stranded RNA is present in extracts of tobacco plants infected with tobacco mosaic virus, *Science* **199**:538.

Derrick, K. S., and Brlansky, R. H., 1976, Assay for viruses and mycoplasmas using serologically specific electron microscopy, *Phytopathology* **66**:815.

Devergne, J. C., Cardin, L., and Quiot, J. B., 1978, Détection et identification sérologique des infections naturelles par le Virus de la Mosaïque due Concombre, *Ann. Phytopathol.* **10**:233.

Doane, F. W., and Anderson, N., 1977, Electron and immunoelectron microscopic procedures for diagnosis of viral infections, in: *Comparative Diagnosis of Viral Diseases*, Vol. II (E. Kurstak and C. Kurstak, eds.), pp. 505–539, Academic Press, New York.

Duffus, J. E., and Gold, A. H., 1969, Membrane feeding and infectivity neutralization used in a serological comparison of potato leaf roll and beet western yellows viruses, *Virology* **37**:150.

Duffus, J. E., and Russell, G. E., 1975, Serological relationship between beet western yellows and beet mild yellowing viruses, *Phytopathology* **65**:811.

Dulbecco, R., and Vogt, M., 1954, Plaque formation and isolation of pure lines with poliomyelitis viruses, *J. Exp. Med.* **99**:167.

Dulbecco, R., Vogt, M., and Strickland, A. G. R., 1956, A study of the basic aspects of neutralization of two animal viruses, western equine encephalitis virus and poliomyelitis virus, *Virology* **2**:162.

Dunlap, R. C., and Barker, L. F., 1973, Vaccinia virus strain differences. I. Neutralization of vaccinia virus strains by homologous and heterologous antisera, *Arch. Gesamte Virusforsch.* **42**:107.

Edwards, E. A., Valters, W. A., Boehm, L. G., and Rosenbaum, M. J., 1975, Visualization by immune electron microscopy of viruses associated with acute respiratory disease, *J. Immunol. Methods* **8**:159.

Ellens, D. J., and De Leeuw, P. W., 1977, Enzyme-linked immunosorbent assay for diagnosis of rotavirus infections in calves, *J. Clin. Microbiol.* **6**:530.

Emmons, R. W., and Riggs, J. L. 1977, Application of immunofluorescence to diagnosis of viral infections, in: *Methods in Virology*, Vol. VI (K. Maramorosch and H. Koprowski, eds.), pp. 1–28, Academic Press, New York.

Engvall, E., and Perlmann, P., 1971, Enzyme-linked immunosorbent assay (ELISA). Quantitative assay of immunoglobulin G, *Immunochemistry* **8**:871.

Enlander, D., Dos Remedios, L. V., Weber, P. M., and Drew, L., 1976, Radioimmunoassay for herpes simplex virus, *J. Immunol. Meth.* **10**:357.

Eppstein, D. A., and Thoma, J. A., 1977, Characterization and serology of the matrix protein from a nuclear-polyhedrosis virus of *Trichoplusia ni* before and after degradation by an endogenous proteinase, *Biochem. J.* **167**:321.

Esposito, J. J., Obijeski, J. L., and Nakano, J. H., 1977, Serological relatedness of monkeypox, variola, and vaccinia viruses, *J. Med. Virol.* **1**:35.

Faulk, W. P., Vyas, G. N., Phillips, C. A., Fudenberg, H. H., and Chism, K., 1971, Passive hemagglutination test for anti-rhinovirus antibodies, *Nature* (*London*) *New Biol.* **231**:101.

Faure, M., Dupouey, P., and Morelec, M. J., 1977, *Les Techniques de l'Immunofluorescence et les Réactions Immuno-enzymatiques*, Maloine, Paris.

Fauvel, M., Artsob, H., and Spence, L., 1977, Immune electron microscopy of arboviruses, *Am. J. Trop. Med. Hyg.* **26**:798.

Favre, M., Breitburd, F., Croissant, O., and Orth, G., 1974, Hemagglutinating activity of bovine papilloma virus, *Virology* **60**:572.

Fazekas de St. Groth, S., 1962, The neutralization of viruses, *Adv. Virus Res.* **9**:1.

Fazekas de St. Groth, S., and Webster, R. G., 1963, The neutralization of animal viruses. IV. Parameters of the influenza virus–antibody system, *Immunology* **90**:151.

Feinstone, S. M., Kapikan, A. Z., and Purcell, R. H., 1973, Hepatitis A: Detection by immune electron microscopy of a viruslike antigen associated with acute illness, *Science* **182**:1026.

Feldman, M., and Basten, A., 1971, The relationship between antigenic structure and the requirement for thymus derived cells in the immune response, *J. Exp. Med.* **134**:103.

Fiala, M., 1969, A study of the neutralization of rhinoviruses by means of a plaque-reduction test, *J. Immunol.* **103**:107.

Forghani, B., Schmidt, N. J., and Lennette, E. H., 1975, Solid phase radioimmunoassay for typing herpes simplex viral antibodies in human sera, *J. Clin. Microbiol.* **2**:410.

Forman, A. J., 1974, A study of foot-and-mouth disease virus strains by complement fixation. II. A comparison of tube and microplate tests for the differentiation of strains, *J. Hyg.* **72**:407.

Francki, R. I. B., 1972, Purification of viruses, in: *Principles and Techniques in Plant Virology* (C. I. Kado and H. O. Agrawal, eds.), pp. 295–335, Van Nostrand Reinhold, New York.

Franklin, N. C., 1961, Serological study of tail structure and function in coliphages T2 and T4, *Virology* **14**:419.

Fritz, R. B., and Rivers, S. L., 1972, Hepatitis-associated antigen: Detection by antibody-sensitized latex particles, *J. Immunol.* **108**:108.

Fuchs, E., 1976, Der Nachweis des Virus der Nekrotischen Rübenvergilbung (NRVV) mit dem Latextest, *Arch. Phytopathol. Pflanzenschutz* **12**:355.

Gamez, R., Black, L. M., and MacLeod, R., 1967, Reexamination of the serological relationship between wound tumor virus and reovirus, *Virology* **32**:163.

Gardner, P. S., 1977, Rapid virus diagnosis, *J. Gen. Virol.* **36**:1.

Gardner, P. S., and McQuillin, J., 1974, *Rapid Virus Diagnosis: Application of Immunofluorescence*, Butterworth, London.

Gera, A., Loebenstein, G., and Raccah, B., 1978, Detection of cucumber mosaic virus in viruliferous aphids by enzyme-linked immunosorbent assay, *Virology* **86**:542.

Gerhard, W., Croce, C. M., Lopes, D., and Koprowski, H., 1978, Repertoire of antiviral antibodies expressed by somatic cell hybrids. *Proc. Natl. Acad. Sci. USA* **75**:1510.

Gillette, K. G., Coria, M. F., Boney, Jr., W. A., and Stone, H. D., 1975, Viscerotropic velogenic Newcastle disease in turkeys: Virus shedding and persistence of infection in susceptible and vaccinated poults, *Avian Dis.* **19**:31.

Gipson, T. G., Daniels, C. A., and Notkins, A. L., 1974, Interaction of rheumatoid factor with infectious vaccinia virus–antibody complexes, *J. Immunol.* **112**:2087.

Gocke, D. J., and Howe, C., 1970, Rapid detection of Australia antigen by counter-immunoelectrophoresis, *J. Immunol.* **104**:1031.

Goding, J. W., 1978, Use of staphylococcal protein A as an immunological reagent, *J. Immunol. Methods* **20**:241.

Gold, A. H., 1961, Antihost serum improves plant virus purification, *Phytopathology* **51**:561.

Gold, A. H., and Duffus, J. E., 1967, Infectivity neutralization—A serological method as applied to persistent viruses of beets, *Virology* **31**:308.

Goodman, J. W., and Donch, J. J., 1965, Phage-neutralizing activity in light polypeptide chains of rabbit antibody, *Immunochemistry* **2**:351.

Grandien, M., 1977, Evaluation of tests for rabies antibody and analysis of serum responses after administration of three different types of rabies vaccines, *J. Clin. Microbiol.* **5**:263.

Grandien, M., and Norrby, E., 1975, Characterization of adenovirus antibodies by single radial diffusion in agarose gels containing immobilized intact virus particles, *J. Gen. Virol.* **27**:343.

Granett, A. L., and Shalla, T. A., 1970, Discrepancies in the intracellular behavior of three strains of tobacco mosaic virus, two of which are serologically indistinguishable, *Phytopathology* **60**:419.

Granoff, A., 1965, The interaction of Newcastle disease virus and neutralizing antibody, *Virology* **25**:38.

Grauballe, P. C., Vestergaard, B. F., Hornsleth, A., Leerhoy, J., and Johnsson, T., 1975, Demonstration by immunoelectro-osmophoresis of precipitating antibodies to a purified rubella virus antigen, *Infect. Immun.* **12**:55.

Grauballe, P. C., Genner, J., Meyling, A., and Hornsleth, A., 1977, Rapid diagnosis of rotavirus infections: Comparison of electron microscopy and immunoelectroosmophoresis for the detection of rotavirus in human infantile gastroenteritis, *J. Gen. Virol.* **35**:203.

Greig, A., 1975, The use of a microtitration technique for the routine assay of African swine fever virus, *Arch. Vriol.* **47**:287.

Grillner, L., and Blomberg, J., 1976, Hemolysis-in-gel and neutralization tests for determination of antibodies to mumps virus, *J. Clin. Microbiol.* **4**:11.

Grist, N. R., Ross, C. A., and Bell, E. J., 1974, *Diagnostic Methods in Clinical Virology*, 2nd ed., Blackwell Scientific Publications, Oxford.

Grogan, R. G., Taylor, R. H., and Kimble, K. A., 1964, The effect of placement of reactants on immunodiffusion precipitin patterns, *Phytopathology* **54**:163.

Gschwender, H. H., and Lehmann-Grube, F., 1975, Micromethod for the titration of lymphocytic choriomeningitis virus in cell cultures, *J. Gen. Virol.* **26**:205.

Gugerli, P., 1978, The detection of two potato viruses by enzyme-linked immunosorbent assay (ELISA), *Phytopathol. Z.* **92**:51.

Gupta, J. D., and Harley, J. D., 1970, Use of formalinized sheep erythrocytes in the rubella hemagglutination-inhibition test, *Appl. Microbiol.* **20**:843.

Habel, K., 1969, Virus neutralization test, in: *Fundamental Techniques in Virology* (K. Habel and N. P. Salzmann, eds.) pp. 288–296, Academic Press, New York.

Hahon, N., 1970, Neutralization of residual infectivity of Venezuelan equine encephalomyelitis virus by anti-gamma globulin, *J. Gen. Virol.* **6**:361.

Hahon, N., Simpson, J., and Eckert, H. L., 1975, Assessment of virus infectivity by the immunofluorescent and immunoperoxidase techniques, *J. Clin. Microbiol.* **1**:324.

Haimovich, J., and Sela, M., 1966, Inactivation of poly-DL-alanyl bacteriophage T4 with antisera specific toward poly-DL-alanine, *J. Immunol.* **97**:338.

Hämmerling, U., 1976, Hybrid antibody markers for electron microscopy, in: *Methods in Immunology and Immunochemistry*, Vol. V (C. A. Williams and M. W. Chase, eds.), pp. 464–482, Academic Press, New York.

Hardie, G., and Van Regenmortel, M. H. V., 1977, Isolation of specific antibody under conditions of low ionic strength, *J. Immunol. Methods* **15**:305.

Harrap, K. A., Payne, C. C., and Robertson, J. S., 1977, The properties of three baculoviruses from closely related hosts, *Virology* **79**:14.

Havranek, P., 1978*a*, Use of quantitative immunoelectrophoresis in cucumber mosaic virus assay. I. Identification of antigenic patterns, *Phytopathol. Z.* **92**:351.

Havranek, P., 1978*b*, Use of quantitative immunoelectrophoresis in cucumber mosaic virus assay. II. Strain- and isolate-specific modifications of the antigenic complex, *Phytopathol. Z.* **93**:5.

Herbert, W. J., 1978, Mineral-oil adjuvants and the immunization of laboratory animals in: *Handbook of Experimental Immunology*, Appendix 3 (D. M. Weir, ed.), Blackwell, Oxford.

Herrmann, J. E., Morse, S. A., and Collins, M. F., 1974, Comparison of techniques and immunoreagents used for indirect immunofluorescence and immunoperoxidase identification of enteroviruses, *Infect. Immun.* **10**:220.

Hierholzer, J. C., and Dowdle, W. R., 1970, Immunological basis of adenovirus 8-9 cross-reaction, *J. Virol.* **6**:782.

Hollinger, F. B., Vorndam, V., and Dreesman, G. R., 1971, Assay of Australia antigen and antibody employing double-antibody and solid-phase radioimmunoassay techniques and comparison with the passive hemagglutination methods, *J. Immunol.* **107**:1099.

Hollings, M. 1975, Recent advances in virus detection and identification by bioassay and serological tests, *Acta Hortic.* **36**:23.

Honess, R. W., and Watson, D. H., 1977, Unity and diversity in the herpesviruses, *J. Gen. Virol.* **37**:15.

Hornick, C. L., and Karush, F., 1972, Antibody affinity. III. The role of multivalence, *Immunochemistry* **9**:325.

Horwitz, M. S., and Scharff, M. D., 1969*a*, The production of antiserum against viral antigens, in: *Fundamental Techniques in Virology* (K. Habel and N. P. Salzmann, eds.), pp. 253–262, Academic Press, New York.

Horwitz, M. S., and Scharff, M. D., 1969*b*, Immunological precipitation of radioac-

tively labeled viral proteins, in: *Fundamental Techniques in Virology* (K. Habel and N. P. Salzmann, eds.), pp. 297–315, Academic Press, New York.

Howe, C., and Lee, L. T., 1972, Virus-erythrocyte interaction, *Adv. Virus Res.* **17**:1.

Howe, C., Morgan, C., and Hsu, K. C., 1969, Recent virologic applications of ferritin conjugates, *Progr. Med. Virol.* **11**:307.

Hsiung, G. D., 1977, Laboratory diagnosis of viral infections: General principles and recent developments, *Mount Sinai J. Med.* **44**:1.

Ihle, J. N., Hanna, M. G., Jr., Schäfer, W., Hunsmann, G., Bolognesi, D. P., and Hüper, G., 1975, Polypeptides of mammalian oncornaviruses. III. Localization of p15 and reactivity with natural antibody, *Virology* **63**:60.

Inouye, S., and Kono, R., 1972, Effects of a modified kaolin treatment on serum, *Appl. Microbiol.* **23**:203.

Iwakata, S., Morissey, L. R., Rhodes, A. J., and Labzoffsky, N. A., 1974, The use of trypsin treated human group O erythrocytes in rubella hemagglutination-inhibition test, *Arch. Gesamte Virusforsch.* **45**:352.

Jackson, D. C., Russell, R. J., Ward, C. W., and Dopheide, T. A., 1978, Antigenic determinants of influenza virus hemagglutinin. I. Cyanogen bromide peptides derived from A/MEMPHIS/72 hemagglutinin possess antigenic activity, *Virology* **89**:199.

Jerne, N. K., and Avegno, P., 1956, The development of the phage-inactivating properties of serum during the course of specific immunization of an animal: Reversible and irreversible inactivation, *J. Immunol.* **76**:200.

Johansson, M. E., Bergquist, N. R., and Grandien, M., 1976, Antibodies to calf serum as a cause of unwanted reaction in immunofluorescence tests, *J. Immunol. Methods* **11**:265.

John, V. T., 1965, A microimmuno-osmophoretic technique for assay of tobacco mosaic virus, *Virology* **27**:121.

Joo, H. S., Donaldson-Wood, C. R., and Johnson, R. H., 1976, A standardized haemagglutination inhibition test for porcine parvovirus antibody, *Austr. Vet. J.* **52**:422.

Juckes, I. R. M. 1971, Fractionation of proteins and viruses with polyethylene glycol, *Biochim. Biophys. Acta* **229**:535.

Juretic, N., and Mamula, D., 1978, Studies in the application of single radial immunodiffusion in the quantitation of several isometric plant viruses, *Phytopathol. Z.* **93**:26.

Kabat, E. A., and Mayer, M. M., 1961, *Experimental Immunochemistry*, 2nd ed. (C. C. Thomas), Springfield, Illinois.

Kapikian, A. Z., Almeida, J. D., and Stott, E. J., 1972*a*, Immune electron microscopy of rhinoviruses, *J. Virol.* **10**:142.

Kapikian, A. Z., Wyatt, R. G., Dolin, R., Thornhill, T. S., Kaliga, A. R., and Chanock, R. M., 1972*b*, Visualization by immune electron microscopy of a 27-nm particle associated with acute infectious nonbacterial gastroenteritis, *J. Virol.* **10**:1075.

Kawamura, A., 1977, *Fluorescent Antibody Techniques and Their Applications*, 2nd ed., Univesrity of Tokyo Press, Tokyo, University Park Press, Baltimore.

Kelen, A. E., Hathaway, A. E., and McLeod, D. A., 1971, Rapid detection of Australia/SH antigen and antibody by a simple and sensitive technique of immunoelectronmicroscopy, *Can. J. Microbiol.* **17**:993.

Keller, R., 1965, Reactivation by physical methods of antibody-neutralized poliovirus, *J. Immunol.* **94**:143.

Keller, R., 1966, The stability of neutralization of poliovirus by native antibody and enzymatically derived fragments, *J. Immunol.* **96**:96.

Kelley, J. M., Emerson, S. U., and Wagner, R. R., 1972, The glycoprotein of vesicular stomatitis virus is the antigen that gives rise to and reacts with neutralizing antibody, *J. Virol.* **10**:1231.

Kelly, D. C., Edwards, M. L., and Robertson, J. S., 1978, The use of enzyme-linked immunosorbent assay to detect, and discriminate between small iridescent viruses, *Ann. Appl. Biol.* **90**:369.

Kenny, M. T., Albright, K. L., and Sanderson, R. P., 1970, Microneutralization test for the determination of mumps antibody in Vero cells, *Appl. Microbiol.* **20**:371.

King, J., 1968, Assembly of the tail of bacteriophage T4, *J. Mol. Biol.* **32**:231.

Kiriyama, K., and Ohsumi, H., 1973, Antibody production in tumorous ascites of mice injected with plant viruses, *Ann. Phytopathol. Soc. Jpn.* **39**:318.

Kitces, E. N., Morahan, P. S., Tew, J. G., and Murray, B. K., 1977, Protection from oral herpes simplex virus infection by a nucleic acid-free vaccine, *Infect. Immun.* **16**:955.

Kjellén, L., 1966, Heterologous neutralization of adenovirus type 5 at low pH, *Virology* **28**:492.

Kjellén, L., and Pereira, H. G., 1968, Role of adenovirus antigens in the induction of virus neutralizing antibody, *J. Gen. Virol.* **2**:177.

Kleczkowski, A., 1961, Serological behaviour of tobacco mosaic virus and of its protein fragments, *Immunology* **4**:130.

Kleczkowski, A., 1965, A study of the effects of salt and of pH on precipitation of antigen-antibody compounds, *Immunology* **8**:170.

Kleczkowski, A., 1966, Serological properties of viruses and their fragments, *Proc. Conf. Plant Viruses*, pp. 196–204, Amsterdam, North Holland.

Klingeborn, B., and Dinter, Z., 1973, Equine abortion (herpes) virus: Properties of the hemagglutinin in virus suspensions, *Virology* **56**:164.

Klinman, N. R., Long, C. A., and Karush, F., 1967, The role of antibody bivalence in the neutralization of bacteriophage, *J. Immunol.* **99**:1128.

Koenig, R., 1970, Effect of organic mercury compounds in serological tests on some isometric plant viruses, *J. Gen. Virol.* **7**:257.

Koenig, R., 1976, A loop-structure in the serological classification system of tymoviruses, *Virology* **72**:1.

Koenig, R., 1978, ELISA in the study of homologous and heterologous reactions of plant viruses, *J. Gen. Virol.* **40**:309.

Koenig, R., and Bode, O., 1978, Sensitive detection of Andean potato latent and Andean potato mottle viruses in potato tubers with the serological latex test, *Phytopathol. Z.* **92**:275.

Koenig, R., and Givord, L., 1974, Serological interrelationships in the turnip yellow mosaic virus group, *Virology* **58**:119.

Köhler, G., and Milstein, C., 1975, Continuous cultures of fused cells secreting antibody of predefined specificity, *Nature* (*London*) **256**:495.

Kraehenbuhl, J. P., and Jamieson, J. D., 1976, Enzyme-labeled antibody markers for electron microscopy, in: *Methods in Immunology and Immunochemistry*, Vol. V (C. A. Williams and M. W. Chase, eds.), pp. 482–495, Academic Press, New York.

Kreuter, J., and Speiser, P. P., 1976, New adjuvants on a polymethylmethacrylate base, *Infect. Immun.* **13**:204.

Krummel, W. M., and Uhr, J. W., 1969, A mathematical and experimental study of the kinetics of neutralization of bacteriophage ϕX174 by antibodies, *J. Immunol.* **102**:772.

Kurstak, E., and Kurstak, C., 1977, *Comparative Diagnosis of Viral Diseases*, Vols. 1 and 2, Academic Press, New York.

Kurstak, E., and Morisset, R., 1974, *Viral Immunodiagnosis*, Academic Press, New York.

Kurstak, E., Tyssen, P., and Kurstak, C., 1977, Immunoperoxidase technique in diagnostic virology and research: Principles and applications, in: *Comparative Diagnosis of Viral Diseases*, Vol. II (E. Kurstak and C. Kurstak, eds.), pp. 403–448, Academic Press, New York.

Lafferty, K. J., 1963, The interaction between virus and antibody. II. Mechanism of the reaction, *Virology* **21**:76.

Lafferty, K. J., and Oertelis, S., 1963, The interaction between virus and antibody. III. Examination of virus-antibody complexes with the electron microscope, *Virology* **21**:91.

Laing, A. C., 1974, Comparison of antiglobulin, direct, and anticomplement immunofluorescent staining for the identification of cytomegalovirus in cultured human fibroblasts, *Can. J. Microbiol.* **20**:1353.

Langbeheim, H., Arnon, R., and Sela, M., 1978, Adjuvant effect of a peptidoglycan attached covalently to a synthetic antigen provoking anti-phage antibodies, *Immunology* **35**:573.

Langenberg, W. G., 1974, Leaf-dip serology for the determination of strain relationships of elongated plant viruses, *Phytopathology* **64**:128.

Laurell, C. B., 1966, Quantitative estimation of proteins by electrophoresis in agarose gel containing antibodies, *Anal. Biochem.* **15**:45.

Laver, W. G., 1973, The polypeptides of influenza viruses, *Adv. Virus Res.* **18**:57.

Lee, T. K., Hutchinson, H. D., and Ziegler, D. W., 1977, Comparison of rabies humoral antibody titers in rabbits and humans by indirect radioimmunoassay, rapid-fluorescent-focus-inhibition technique, and indirect fluorescent-antibody assay, *J. Clin. Microbiol.* **5**:320.

Lennette, E. H., and Schmidt, N. J., 1969, *Diagnostic Procedures for Viral and Rickettsial Infections*, 4th ed., American Public Health Association, Inc., New York.

Levine, L., and Van Vunakis, H., 1967, Micro complement fixation, *Methods Enzymol.* **11**:928.

Liebhaber, H., 1970, Measurement of rubella antibody by hemagglutination inhibition. II. Characteristics of an improved HAI test employing a new method for the removal of non-immunoglobulin HA inhibitors from serum, *J. Immunol.* **104**:826.

Linscott, W. D., and Levinson, W. E., 1969, Complement components required for virus neutralization by early immunoglobulin antibody, *Proc. Natl. Acad. Sci. USA* **64**:520.

Lister, R. M., and Rochow, W. F., 1979, Detection of barley yellow dwarf virus by enzyme-linked immunosorbent assay, *Phytopathology* **69**:649.

Litwin, S. D., 1977, Latex fixation techniques, in: *Methods in Immunology and Immunochemistry*, Vol. 4 (C. A. Williams and M. W. Chase, eds.), p. 115, Academic Press, New York.

Luisoni, E., Milne, R. G., and Boccardo, G., 1975, The maize rough dwarf virion. II. Serological analysis, *Virology* **68**:86.

Lwoff, A., and Tournier, P., 1971, Remarks on the classification of viruses, in: *Comparative Virology* (K. Maramorosch and E. Kurstak, eds.), pp. 1–42, Academic Press, New York.

Maat, D. Z., 1970, Experiments on the use of latex, bentonite, and water-insoluble antiserum protein polymers in sensitive serological tests, *Neth. J. Plant. Pathol.* **76**:79.

Majer, M., 1972, Virus sensitization, *Curr. Top. Microbiol. Immunol.* **58**:69.

Majer, M., and Link, F., 1970, Studies on the non-neutralizable fraction of vaccinia virus, *Clin. Exp. Immunol.* **7**:283.

Mancini, G., Carbonara, A. O., and Heremans, J. F., 1965, Immunochemical quantitation of antigens by single radial immunodiffusion, *Immunochemistry* **2**:235.

Mandel, B., 1961, Reversibility of the reaction between poliovirus and neutralizing antibody of rabbit origin, *Virology* **14**:316.

Mandel, B., 1971, Characterization of type I poliovirus by electrophoretic analysis, *Virology* **44**:554.

Mandel, B., 1976, Neutralization of poliovirus: A hypothesis to explain the mechanism and the one-hit character of the neutralization reaction, *Virology* **69**:500.

Mandel, B., 1979, Interaction of viruses with neutralizing antibodies, in: *Comprehensive Virology*, Vol. 15 (H. Fraenkel-Conrat and R. Wagner, eds.), pp. 37–121, Plenum Press, New York.

Martin, M. L., Palmer, E. L., and Kissling, R. E., 1972, Complement-fixing antigens of herpes simplex virus types 1 and 2: Reactivity of capsid, envelope and soluble antigens, *Infect. Immun.* **5**:248.

Matthews, R. E. F., 1967, Serological techniques for plant viruses, in: *Methods in Virology*, Vol. III (K. Maramorosch and H. Koprowski, eds.), pp. 199–241, Academic Press, New York.

Mautner, V., and Willcox, H. N. A., 1974, Adenovirus antigens: A model system in mice for subunit vaccination, *J. Gen. Virol.* **25**:325.

Mayr, A., Bachmann, P. A., Bibrack, B., and Wittmann, G., 1977, *Virologische Arbeitsmethoden*, Vol. II: *Serologie*, Gustav Fischer, Stuttgart.

Mazzone, H. M., and Tignor, G. H., 1976, Insect viruses: Serological relationships, *Adv. Virus Res.* **20**:237.

McBride, W. D., 1959, Antigenic analysis of polioviruses by kinetic studies of serum neutralization, *Virology* **7**:45.

McVicar, J. W., Sutmoller, P., and Andersen, A. A., 1974, Foot-and-mouth disease virus: Plaque reduction neutralization, *Arch. Gesamte Virusforsch.* **44**:168.

Middleton, P. J., Petric, M., Hewitt, C. M., Szymanski, M. T., and Tam, J. S., 1976, Counter-immunoelectro-osmophoresis for the detection of infantile gastroenteritis virus (orbi-group) antigen and antibody, *J. Clin. Pathol.* **29**:191.

Middleton, P. J., Holdaway, M. D., Petric, M., Szymanski, M. T., and Tam, J. S., 1977, Solid-phase radioimmunoassay for the detection of rotavirus, *Infect Immun.* **16**:439.

Miller, J. M., and Van der Maaten, M. J., 1977, Use of glycoprotein antigen in the immunodiffusion test for bovine leukemia virus antibodies, *Eur. J. Cancer* **13**: 1369.

Mills, K. W., Gerlach, E. H., Bell, J. W., Farkas, M. E., and Taylor, R. J., 1978, Serotyping herpes simplex virus isolates by enzyme-linked immunosorbent assays, *J. Clin. Microbiol.* **7**:73.

Milne, R. G., and Lesemann, D. E., 1978, An immunoelectron microscopic investigation of oat sterile dwarf and related viruses, *Virology* **90**:299.

Milne, R. G., and Luisoni, E., 1977, Rapid immune electron microscopy of virus preparations, in: *Methods in Virology*, Vol. VI (K. Maramorosch and H. Koprowski, eds.), pp. 265–281, Academic Press, New York.

Milton, R. C., de L., and Van Regenmortel, M. H., V., 1979, Immunochemical studies of tobacco mosaic virus. III. Demonstration of five antigenic regions in the protein subunit, *Mol. Immunol.* **16**:179.

Monath, T. P. C., Lindsey, H. S., Nuckolls, J. G., Chappell, W. A., and Henderson, B. E., 1970, Comparison of methods for removal of nonspecific inhibitors or arbovirus hemagglutination, *Appl. Microbiol.* **20**:748.

Morein, B., Helenius, A., Simons, K., Pettersson, R., Kääriäinen, L., and Schirrmacher, V., 1978, Effective subunit vaccines against an enveloped animal virus, *Nature* (*London*) **276**:715.

Mostow, S. R., Schild, G. C., Dowdle, W. R., and Wood, R. J., 1975, Application of the single radial diffusion test for assay of antibody to influenza type A viruses, *J. Clin. Microbiol.* **2**:531.

Moyer, J.W., and Smith, S. H., 1977, Purification and serological detection of mushroom viruslike particles, *Phytopathology* **67**:1207.

Munro, T. F., and Wooley, R. E., 1973, Neutralization kinetics study of selected reoviruses, *Infect. Immun.* **8**:628.

Nairn, R. C., 1975, *Fluorescent Protein Tracing*, Churchill Livingstone, London.

Naso, R. B., Arcement, L. J., and Arlinghaus, R. B., 1975, Biosynthesis of Rauscher leukemia viral proteins, *Cell* **4**:31.

Nelson, D. B., Quirin, E. P., and Inhorn, S. L., 1972, Improved dextran sulfate-calcium chloride method for the removal of nonspecific inhibitors with modifications for nonspecific agglutinin removal in the rubella hemagglutination inhibition test, *Appl. Microbiol.* **24**:264.

Neurath, A. R., and Rubin, B. A., 1971, *Viral Structural Components as Immunogens of Prophylactic Value*, Karger, Basel.

Neurath, A. R., Trepo, C., Chen, M., and Prince, A. M., 1976, Identification of additional antigenic sites on dane particles and the tubular forms of hepatitis B surface antigen, *J. Gen. Virol.* **30**:277.

Neurath, A. R., Strick, N., and Huang, C. Y., 1978, Properties of delipidated hepatitis B surface antigen (HBsAg) and preparation of its proteolytic cleavage fragments carrying HBs-Ag-specific antigenic determinants, *Intervirology* **10**:265.

Nicolaïeff, A., and Van Regenmortel, M.H.V., 1980, Specificity of trapping of plant viruses on antibody-coated electron microscope grids, *Ann. Virol.* **131E**:95.

Nicolaïeff, A., Obert, G., and Van Regenmortel, M. H., V., 1980, Detection of rota-virus by serological trapping on antibody-coated electron microscope grids, *J. Clin. Microbiol.* **12**:101.

Norrby, E., 1968, Biological significance of structural adenovirus components, *Curr. Top. Microbiol. Immunol.* **43**:1.

Norrby, E., 1969, The relationship between the soluble antigens and the virion of adenovirus type 3. IV. Immunological complexity of soluble components, *Virology* **37**:565.

Norrby, E., and Wadell, G., 1972, The relationship between the soluble antigens and the virion of adenovirus type 3. VI. Further characterization of antigenic sites available at the surface of virions, *Virology* **48**:757.

Norrby, E., Grandien, M., and Orvell, C., 1977, New tests for characterization of mumps virus antibodies: Hemolysis inhibition, single radial immunodiffusion with immobilized virions, and mixed hemadsorption, *J. Clin. Microbiol.* **5**:346.

Norrild, B., Bjerrum, O. J., and Vestegaard, B. F., 1977, Polypeptide analysis of individual immunoprecipitates from crossed immunoelectrophoresis, *Anal. Biochem.* **81**:432.

Notkins, A. L., 1971, Infectious virus-antibody complexes: Interaction with anti-immunoglobulins, complement and rheumatoid factors, *J. Exp. Med.* **134**:41.

Oldstone, M. B. A., 1975, Virus neutralization and virus-induced immune complex disease, *Progr. Med. Virol.* **19**:84.

Oldstone, M. B., Cooper, N. R., and Larson, D. L., 1974, Formation and biological role of polyoma virus–antibody complexes: A critical role for complement, *J. Exp. Med.* **140**:549.

Oroszlan, S., and Gilden, R. V., 1970, Immune virolysis: Effect of antibody and complement on C-type RNA virus, *Science* **168**:1478.

Oshiro, L. S., Levy, J. A., Riggs, J. L., and Lennette, E. H., 1977, Distinction between envelope antigens of murine xenotropic and ecotropic type C viruses by immunoelectron microscopy, *J. Gen. Virol.* **35**:317.

Osterrieth, P. M., 1972, La neutralisation des virus, *Bull. Inst. Pasteur Paris* **70**:281.

Otsuki, Y., and Takebe, I., 1978, Production of mixedly coated particles in tobacco mesophyll protoplasts doubly infected by strains of tobacco mosaic virus, *Virology* **84**:162.

Ouchterlony, Ö., 1968, *Handbook of Immunodiffusion and Immunoelectrophoresis*, Ann Arbor Scientific Publications, Ann Arbor, Mich.

Ouchterlony, Ö., and Nilsson, L. A., 1978, Immunodiffusion and immunoelectrophoresis, in: *Handbook of Experimental Immunology*, 3rd ed. (D. M. Weir, ed.), Chap. 19, Blackwell, Oxford.

Oxford, J. S., and Schild, G. C., 1976, Immunological and physicochemical studies of influenza matrix (M) polypeptides, *Virology* **74**:394.

Oxford, J. S., and Schild, G. C., 1977, Quantification of influenza virus structural proteins using rocket immunoelectrophoresis, *J. Gen. Virol.* **38**:187.

Paliwal, Y. C., 1977, Rapid diagnosis of barley yellow dwarf virus in plants using serologically specific electron microscopy, *Phytopathol. Z.* **89**:25.

Parks, W. P., and Rapp, F., 1975, Prospects for herpesvirus vaccination—Safety and efficacy considerations, *Progr. Med. Virol.* **21**:188.

Penny, J. B., and Narayan, O., 1973, Studies of the antigenic relationships of the new human papovaviruses by electron microscopy agglutination, *Infect. Immun.* **8**: 299.

Peters, J. H., and Coons, A. H., 1976, Fluorescent antibody as specific cytochemical reagents, in: *Methods in Immunology and Immunochemistry*, Vol. 5 (C. A. Williams and M. W. Chase, eds.), pp. 424–444 Academic Press, New York.

Pettersson, U., 1971, Structural proteins of adenoviruses. VI. On the antigenic determinants of the hexon, *Virology* **43**:123.

Philipson, L., 1966, Interaction between poliovirus and immunoglobulins. II. Basic aspects of virus–antibody interaction, *Virology* **28**:35.

Polley, J. R., 1977, Viral diagnostic reagents, in: *Comparative Diagnosis of Viral Diseases*, Vol. II (E. Kurstak and C. Kurstak, eds.,), pp. 347–402, Academic Press, New York.

Polson, A., 1971, Determination of diffusion coefficients by a method of double diffu-

sion in gels, in: *Methods in Immunology and Immunochemistry*, Vol. 3 (C. A. Williams and M. W. Chase, eds.), pp. 180–186, Academic Press, New York.

Polson, A., and Russell, B., 1967, Electrophoresis of viruses, in: *Methods in Virology*, Vol. 2 (K. Maramorosch and H. Koprowski, eds.), pp. 391–426, Academic Press, New York.

Polson, A., Keen, A., Sinclair-Smith, C., and Furminger, I. G., S., 1972, Polyethylene glycol purification of influenza virus with respect to aggregation and antigenicity, *J. Hyg.* **70**:255.

Polson, A., Von Wechmar, B., and Moodie, J. W., 1978, Preparative immunoabsorption electrophoresis, *Immunol. Commun.* **7**:91.

Potgieter, L. N. D., and Maré, C. J., 1974, Differentiation of strains of infectious bovine rhinotracheitis virus by neutralization kinetics with late 19 S rabbit antibodies, *Infect. Immun.* **10**:520.

Prager, E. M., and Wilson, A. C., 1971, The dependence of immunological cross-reactivity upon sequence resemblance among lysozymes. II. Comparison of precipitin and micro-complement fixation results, *J. Biol. Chem.* **246**:7010.

Prakash, V. J., Norrby, E., and Payne, L., 1977, Single radial immunodiffusion test for detecting antibodies against surface antigens of intracellular and extracellular vaccinia virus, *J. Gen. Virol.* **35**:465.

Purcifull, D. E., and Batchelor, D. L., 1977, Immunodiffusion tests with sodium dodecyl sulfate (SDS)-treated plant viruses and plant viral inclusions, *Univ. Fl. Agric. Exper. Stn. Tech. Bull. No. 788*, 39 pp.

Purcifull, D. E., and Gooding, G. V. Jr., 1970, Immunodiffusion tests for potato Y and tobacco etch viruses, *Phytopathology* **60**:1036.

Purcell, R. H., Wong, D. C., Moritsugu, Y., Dienstag, J. L., Routernberg, J. A., and Boggs, J. D., 1976, A microtiter solid phase radioimmunoassay for hepatitis A antigen and antibody, *J. Immunol.* **116**:349.

Quirin, E. P., Nelson, D. B., and Inhorn, S. L., 1972, Use of trypsin-modified human erythrocytes in rubella hemagglutination-inhibition testing, *Appl. Microbiol.* **24**:353.

Radwan, A. I., and Burger, D., 1973, The complement-requiring neutralization of equine arteritis virus by late antisera, *Virology* **51**:71.

Radwan, A. I., and Crawford, T. B., 1974, The mechanism of neutralization of sensitized equine arteritis virus by complement components, *J. Gen. Virol.* **25**:229.

Ragetli, H. W. J., and Weintraub, M., 1964, Immuno-osmophoresis, a rapid and sensitive method for evaluating viruses, *Science* **144**:1023.

Rapp, R., and Reed, C., 1976, Experimental evidence for the oncogenic potential of herpes simplex viruses, *Cancer Res.* **36**:800.

Rappaport, I., 1970, An analysis of the inactivation of MS2 bacteriophage with antiserum, *J. Gen. Virol.* **6**:25.

Reinganum C., and Scotti, P. D., 1976, Serological relations between twelve small RNA viruses of insects, *J. Gen. Virol.* **31**:131.

Reno, P. W., and Hoffmann, E. M., 1972, Enhancement of hemagglutination inhibition by complement, *Infect Immun.* **6**:945.

Richter, J., 1967, Die Anwendung des Hämagglutinationstestes nach BOYDEN zum Nachweis eines pflanzenpathogenen Virus, *Zbl. Bakt. Abt. II* **121**:269.

Richter, J., 1971, Zur Frage der Charakterisierung von Antiseren gegen pflanzenpathogene Viren und ihrer Eignung für den Virusnachweis mittels passiver Hämagglutination, *Arch. Pflanzenschutz* **7**:189.

Richter, J., Haack, I., and Spaar, D., 1976, Quantitative Bestimmung isometrischer Pflanzen-Viren mit Hilfe des Radialimmundiffusiontestes, *Arch. Phytopathol. Pflanzenschutz* **12**:235.

Rifkind, R. A., 1976, Ferritin-conjugated antibody markers for electron microscopy, in: *Methods in Immunology and Immunochemistry*, Vol. V (C. A. Williams and M. W. Chase, eds.), pp. 458–463, Academic Press, New York.

Robinson, O. R., Jr., Shibley, G. P., and Sevoian, M., 1977, Quantitative immunoelectrophoretic assay for murine oncornavirus p30: Noncovalent facilitation by sodium dodecyl sulfate, *Infect. Immun.* **18**:60.

Rochow, W. F., and Ball, E. M., 1967, Serological blocking of aphid transmission of barley yellow dwarf virus, *Virology* **33**:359.

Rochow, W. F., and Carmichael, L. E., 1979, Specificity among barley yellow dwarf viruses in enzyme immunosorbent assays, *Virology* **95**:415.

Rochow, W. F., and Duffus, J. E., 1978, Relationship between barley yellow dwarf and beet western yellows viruses, *Phytopathology* **68**:51.

Rosen, L., 1969, Hemagglutination with animal viruses, in: *Fundamental Techniques in Virology* (K. Habel and N. P. Salzmann, eds.), pp. 276–287, Academic Press, New York.

Rovozzo, G. C., and Burke, C. N., 1973, *A Manual of Basic Virological Techniques*, pp. 94–125, Prentice-Hall, Englewood Cliffs, N.J.

Rowlands, D. J., Sangar, D. V., and Brown, F., 1971, Relationship of the antigenic structure of foot-and-mouth disease virus to the process of infection, *J. Gen. Virol.* **13**:85.

Rubin, B. A., and Tint, H., 1975, The development and use of vaccines based on studies of virus substructures, *Progr. Med. Virol.* **21**:144.

Russel, S. M., McCahon, D., and Beare, A. S., 1975, A single radial haemolysis technique for the measurement of influenza antibody, *J. Gen. Virol.* **27**:1.

Russell, R. J., and Jackson, D. C., 1978, Direct solid-phase radioimmunoassay for measuring antigenic differences between the hemagglutinins of influenza viruses, *J. Immunol. Methods* **22**:201.

Rweyemamu, M. M., Booth, J. C., Parry, N., and Pay, T. W. F., 1977, Neutralization kinetics studies with type SAT 2 foot-and-mouth disease virus strains. II. Antigenic differentiation of vaccine strains, *J. Hyg.* **78**:429.

Saito, Y., and Iwata, Y., 1964, Hemagglutination test for titration of plant virus, *Virology* **22**:426.

Sartorelli, A. C., Fischer, D. S., and Downs, W. G., 1966, Use of sarcoma 180/TG to prepare hyperimmune ascitic fluid in the mouse, *J. Immunol.* **96**:676.

Schade, C., 1971, Der Nachweis des Virus der Nekrotischen und der Chlorotischen Ringfleckenkrankheit in Kirschen mit dem Latextest als Schnellmethode, *Arch. Pflanzenschutz* **7**:207.

Scherrer, R., and Bernard, S., 1977, Application d'une technique immunoenzymologique (ELISA) à la détection du rotavirus bovin et des anticorps dirigés contre lui, *Ann. Microbiol.* **128A**:499.

Schild, G. C., 1970, Studies with antibody to the purified hemagglutinin of an influenza A_0 virus, *J. Gen. Virol.* **9**:191.

Schild, G. C., and Pereira, H. G., 1969, Characterization of the ribonucleoprotein and neuraminidase of influenza A viruses by immunodiffusion, *J. Gen. Virol.* **4**:355.

Schild, G. C., Henry-Aymard, M., and Pereira, H. G., 1972, A quantitative, single-

radial-diffusion test for immunological studies with influenza virus, *J. Gen. Virol.* **16**:231.

Schild, G. C., Pereira, M. S., and Chakraverty, P., 1975, Single-radial-haemolysis: A new method for the assay of antibody to influenza haemagglutinin. Applications for the diagnosis and seroepidemiologic surveillance of influenza, *Bull. WHO* **52**:43.

Schmidt, N. J., and Lennette, E. H., 1971, Comparison of various methods for preparation of viral serological antigens from infected cell cultures, *Appl. Microbiol.* **21**:217.

Schmidt, N. J., and Lennette, E. H., 1973, Advances in the serodiagnosis of viral infections, *Prog. Med. Virol.* **15**:244.

Schmidt, N. J., Lennette, E. H., and Magoffin, R. L., 1969, Immunological relationship between herpes simplex and varicella-zoster viruses demonstrated by complement-fixation, neutralization and fluorescent antibody tests, *J. Gen. Virol.* **4**:321.

Schulze, I. T., 1973, Structure of the influenza virion, *Adv. Virus Res.* **18**:1.

Scott, D. W., 1965, Serological cross reactions among the RNA-containing coliphages, *Virology* **26**:85.

Scott, H. A., 1973, Interpretation of gel diffusion tests, *Phytopathology* **63**:439.

Shanmugam, G., Vecchio, G., Attardi, D., and Green, M., 1972, Immunological studies on viral polypeptide synthesis in cells replicating murine sarcoma-leukemia virus, *J. Virol.* **10**:447.

Shepard, J. F., 1972, Gel diffusion methods for the serological detection of potato viruses X, S and M, *Montana Agric. Exp. Stn. Bull.* **662,** 72 pp.

Shepard, J. F., and Secor, G. A., 1969, Detection of potato virus X in infected plant tissue by radial and double-diffusion tests in agar, *Phytopathology* **59**:1838.

Shepard, J. F., and Shalla, T. A., 1970, An antigenic analysis of potato virus X and of its degraded protein. I. Evidence for and degree of antigenic disparity, *Virology* **42**:825.

Shepard, J. F., Secor, G. A., and Purcifull, D. E., 1974, Immunochemical cross-reactivity between the dissociated capsid proteins of PVY group plant viruses, *Virology* **58**:464.

Shukla, D. D., and Gough, K. H., 1979, The use of protein A, from *Staphylococcus aureus*, in immune electron microscopy for detecting plant virus particles, *J. Gen. Virol.* **45**:533.

Shvartsman, Y. S., and Zykov, M. P., 1976, Secretory anti-influenza immunity, *Adv. Immunol.* **22**:291.

Sibal, L. R., Hollis, V. W., Jr., and Fink, M. A., 1971, Detection of antigenic subunits of a murine leukemia virus by passive hemagglutination, *J. Immunol.* **106**:1050.

Sim, C., and Watson, D. H., 1973, The role of type specific and cross reacting structural antigens in the neutralization of herpes simplex virus types 1 and 2, *J. Gen. Virol.* **19**:217.

Slack, S. A., and Shepherd, R. J., 1975, Serological detection of seed-borne barley stripe mosaic virus by a simplified radial-diffusion technique, *Phytopathology* **65**:948.

Sorensen, K. J., 1974, Microadaptation of hemadsorption inhibition for neutralization tests with hemagglutinating encephalomyelitis virus, *Appl. Microbiol.* **28**:653.

Stannard, L. M., and Moodie, J. W., 1976, Hepatitis B antigen: IgG components shown by immune electron microscopy, *J. Gen. Virol.* **30**:269.

Stavitsky, A. B., 1977, Hemagglutination with tannic acid-treated (tanned) eryth-

rocytes, in: *Methods in Immunology and Immunochemistry*, Vol. 4 (C. A. Williams and M. W. Chase, eds.), p. 30, Academic Press, New York.

Stemke, G. W., Knight, W., Burtz, E., and Gates, D. J., 1974, The mechanism of bacteriophage T4 neutralization: Effects of univalent antibody fragments on T4 adsorption kinetics, *Can. J. Microbiol.* **20**:499.

Ströbel, G., 1974, Restoration of the immune response to tobacco mosaic virus with homologous and heterologous thymus extracts in thymocyte-depleted mice, *Eur. J. Immunol.* **4**:621.

Styk, B., and Schmidt, N. J., 1968, Enterovirus immunodiffusion reactions: A comparison of results obtained in agarose and agar using two different microdiffusion systems, *J. Immunol.* **100**:1223.

Sugiura, T., and Nakajima, H., 1977, Purification of equine infectious anemia virus antigen by affinity chromatography, *J. Clin. Microbiol.* **5**:635.

Svehag, S. E., 1963, Reactivation of neutralized virus by fluorocarbon: Mechanism of action and demonstration of reduced reactivability with time of virus-antibody interaction, *Virology* **21**:174.

Svehag, S. E., 1968, Formation and dissociation of virus-antibody complexes with special reference to the neutralization process, *Prog. Med. Virol.* **10**:1.

Takabayashi, K., and McIntosh, K., 1973, Effect of heat-labile factors on the neutralization of vaccinia virus by human sera, *Infect. Immun.* **8**:582.

Tauraso, N. M., Pedreira, F. A., Spector, S. L., and Bernier, G. M., 1971, Effect of various methods of removing non-specific inhibitors to virus hemagglutination upon serum proteins and immunoglobulins, *Arch. Gesamte Virusforsch.* **34**:214.

Thouvenot, D., Viac, J., Gaudin, O. G., and Sohier, R., 1973, Reproductibilité de la neutralisation cinétique (Test de McBride) pour la différenciation intratypique des poliovirus type I, *Ann. Microbiol.* **124B**:217.

Thresh, J. M., Adams, A. N., Barbara, D. J., and Clark, M. F., 1977, The detection of three viruses of hop (*Humulus lupulus*) by enzyme-linked immunosorbent assay (ELISA), *Ann. Appl. Biol.* **87**:57.

Toms, G. L., and Mostratos, A., 1973, The use of rivanol for the removal of non-specific inhibitors of rubella virus haemagglutinin, *J. Med. Microbiol.* **6**:42.

Tosi, M., and Anderson, D. L., 1973, Antigenic properties of bacteriophage $\phi 29$ structural proteins, *J. Virol.* **12**:1548.

Trautman, R., 1976, Unified mass-action theory for virus neutralization and radioimmunology, *Scand. J. Immunol.* **5**:609.

Trautman, R., and Harris, W. F., 1977, Modeling and computer simulation approach to the mechanism of foot-and-mouth disease virus neutralization assays, *Scand. J. Immunol.* **6**:831.

Tsuchizaki, T., Sasaki, A., and Saito, Y., 1978, Purification of citrus tristeza virus from diseased citrus fruits and the detection of the virus in citrus tissues by fluorescent antibody techniques, *Phytopathology* **68**:139.

Tyrrell, D. A. J., 1978, Immunological methods in virology, in: *Handbook of Experimental Immunology* (D. M. Weir, ed.), Chapter 40, Blackwell, Oxford.

Tzianabos, T., Herbert, G. A., and White, L. A., 1976, Preparation of rabies fluorescein isothiocyanate-labeled immune globulin from mouse hyperimmune ascitic fluids, *J. Clin. Microbiol.* **3**:609.

Uyemoto, J. K., Grogan, R. G., and Wakeman, J. R., 1968, Selective activation of satellite virus strains by strains of tobacco necrosis virus, *Virology* **34**:410.

Valentine, R. C., and Pereira, H. G., 1965, Antigens and structure of the adenovirus, *J. Mol. Biol.* **13**:13.

Van Regenmortel, M. H. V., 1959, Determination of diffusion coefficient of turnip yellow mosaic virus by the gel precipitin technique, *Biochim. Biophys. Acta* **34**:553.

Van Regenmortel, M. H. V., 1963, Serologically related plant contaminants in preparations of partially purified plant viruses, *Virology* **21**:657.

Van Regenmortel, M. H. V., 1964, Purification of plant viruses by zone electrophoresis, *Virology* **23**:495.

Van Regenmortel, M. H. V., 1966, Plant virus serology, *Adv. Virus Res.* **12**:207.

Van Regenmortel, M. H. V., 1967, Serological studies on naturally occurring strains and chemically induced mutants of tobacco mosaic virus, *Virology* **31**:467.

Van Regenmortel, M. H. V., 1972, Electrophoresis, in: *Principles and Techniques in Plant Virology* (C. I. Kado and H. O. Agrawal, eds.), pp. 390–412, Van Nostrand Reinhold, New York.

Van Regenmortel, M. H. V., 1975, Antigenic relationships between strains of tobacco mosaic virus, *Virology* **64**:415.

Van Regenmortel, M. H. V., 1978, Applications of plant virus serology, *Annu. Rev. Phytopathol.* **16**:57.

Van Regenmortel, M. H. V., and Burckard, J., 1980, Detection of a wide spectrum of tobacco mosaic virus strains by indirect enzyme linked immunosorbent assay (ELISA), *Virology* **106**.

Van Regenmortel, M. H. V., and Hardie, G., 1976, Immunochemical studies of tobacco mosaic virus. II. Univalent and monogamous bivalent binding of IgG antibody, *Immunochemistry* **13**:503.

Van Regenmortel, M. H. V., and Lelarge, N., 1973, The antigenic specificity of different states of aggregation of tobacco mosaic virus protein, *Virology* **52**:89.

Van Regenmortel, M. H. V., and Von Wechmar, M. B., 1970, A re-examination of the serological relationship between tobacco mosaic virus and cucumber virus 4, *Virology* **41**:330.

Van Slogteren, D. H. M., 1969, Analytical serology of plant viruses (Phytophagineae), in: *Analytical Serology of Microorganisms*, Vol. 1 (J. B. G. Kwapinski, ed.), pp. 353–409, John Wiley, New York.

Van Slogteren, D. H. M., 1976, A single immunodiffusion drop test for the detection of lily symptomless virus, *Ann. Appl. Biol.* **82**:91.

Van Weemen, B. K., and Kacaki, J., 1976, A modified haemagglutination inhibition test for rubella antibodies, using standardized, freeze-dried reagents. Report of a comparative multi-centre trial, *J. Hyg.* **77**:31.

Van Weemen, B. K., and Schuurs, A. H. W. M., 1971, Immunoassay using antigen-enzyme conjugates, *FEBS Lett.* **15**:232.

Van Zaane, D., Gielkens, A. L. J., Dekker-Michielsen, M. J. A., and Bloemers, H. P. J., 1975, Virus-specific precursor polypeptides in cells infected with Rauscher leukemia virus, *Virology* **67**:544.

Venekamp, J. H., 1972, Chromatographic purification of plant viruses, in: *Principles and Techniques in Plant Virology* (C. I. Kado and H. O. Agrawal, eds.), pp. 369–389, Van Nostrand Reinhold, New York.

Vestergaard, B. F., 1975, Production of antiserum against a specific herpes simplex virus type 2 antigen, *Scand. J. Immunol.* Suppl. **4(2)**:203.

Vestergaard, B. F., Bjerrum, O. J., Norrild, B., and Grauballe, P. C., 1977, Crossed immunoelectrophoretic studies of the solubility and immunogenicity of herpes simplex virus antigens, *J. Virol.* **24**:82.

Vogt, V. M., and Eisenman, R., 1973, Identification of a large polypeptide precursor of avian oncornavirus proteins, *Proc. Natl. Acad. Sci. USA* **70**:1734.

Voller, A., and Bidwell, D. E., 1977, Enzyme immunoassays and their potential in diagnostic virology, in: *Comparative Diagnosis of Viral Diseases*, Vol. II (E. Kurstak and C. Kurstak, eds.), pp. 449–457, Academic Press, New York.

Voller, A., Bidwell, D., Huldt, G., and Engvall, E., 1974, A microplate method of enzyme-linked immunosorbent assay and its application to malaria, *Bull. WHO* **51**:209.

Voller, A., Bartlett, A., Bidwell, D. E., Clark, M. F., and Adams, A. N., 1976, The detection of viruses by enzyme-linked immunosorbent assay (ELISA), *J. Gen. Virol.* **33**:165.

Von Wechmar, M. B., and Van Regenmortel, M. H. V., 1968, Serological studies on bromegrass mosaic virus and its protein fragments, *Virology* **34**:36.

Vyas, G. N., and Shulman, N. R., 1970, Hemagglutination assay for antigen and antibody associated with viral hepatitis, *Science* **170**:332.

Wadell, G., 1972, Sensitization and neutralization of adenovirus by specific sera against capsid subunits, *J. Immunol.* **108**:622.

Wagner, G. G., Cowan, K. M., and McVicar, J. W., 1972, Screening of sera for antibodies to foot-and-mouth disease viral antigens by radial immunodiffusion, *Infect. Immun.* **5**:227.

Wagner, M., 1973, Method of labeling antibodies for electron microscopic localization of antigens, *Res. Immunochem. Immunobiol.* **3**:185.

Wallis, C., and Melnick, J. L., 1967, Virus aggregation as the cause of the nonneutralizable persistent fraction, *J. Virol.* **1**:478.

Wallis, C., and Melnick, J. L., 1970, Herpesvirus neutralization: Induction of the persistent fraction by insufficient antibody, *Virology* **42**:128.

Warrington, R. E., and Kawakami, Y., 1972, Passive hemagglutination-inhibition test for typing foot-and-mouth disease virus, *Appl. Microbiol.* **23**:98.

Webster, R. G., and Laver, W. G., 1967, Preparation and properties of antibody directed specifically against the neuraminidase of influenza virus, *J. Immunol.* **99**:49.

Webster, R. G., and Laver, W. G., 1975, Antigenic variation of influenza viruses, in: *The Influenza Viruses and Influenza* (E. D. Kilbourne, ed.), pp. 270, Academic Press, New York.

Weeke, B., 1973*a*, Rocket immunoelectrophoresis, *Scand. J. Immunol. Suppl.* **2(1)**:37.

Weeke, B., 1973*b*, Crossed immunoelectrophoresis, *Scand. J. Immunol. Suppl.* **2(1)**47.

Wetter, C., 1965, Serology in virus disease diagnosis, *Annu. Rev. Phytopathol.* **3**:19.

Wetter, C., 1967, Immunodiffusion of tobacco mosaic virus and its interaction with agar, *Virology* **31**:498.

Wetter, C., and Luisoni, E., 1969, Precipitin, agar gel diffusion, and intragel adsorption tests with three strains of tomato bushy stunt virus, *Phytopathol. Z.* **65**:231.

Wheeler, C. R., Briggaman, R. A., and Henderson, R. R., 1969, Discrimination between two strains (types) of herpes simplex virus by various modifications of the neutralization test, *J. Immunol.* **102**:1179.

Wicker, R., 1971, Comparison of immunofluorescent and immunoenzymatic techniques applied to the study of viral antigens, *Ann. N.Y. Acad. Sci.* **177**:490.

Wicker, R., and Avrameas, S., 1969, Localization of virus antigens by enzyme-labeled antibodies, *J. Gen. Virol.* **4**:465.

Wiktor, T. J., and Koprowski, H., 1978, Monoclonal antibodies against rabies virus produced by somatic cell hybridization: Detection of antigenic variants, *Proc. Natl. Acad. Sci. USA* **75**:3938.

Wiktor, T. J., György, E., Schlumberger, H. D., Sokol, F., and Koprowski, H., 1973, Antigenic properties of rabies virus components, *J. Immunol.* **110**:269.

Willcox, N., and Mautner, V., 1976*a*, Antigenic determinants of adenovirus capsids. I. Measurement of antibody cross-reactivity, *J. Immunol.* **116**:19.

Willcox, N., and Mautner, V., 1976*b*, Antigenic determinants of adenovirus capsids. II. Homogeneity of hexons, and accessibility of their determinants, in the virion, *J. Immunol.* **116**:25.

Williams, C. A., 1971, Immunoelectrophoretic analysis in agar gels, in: *Methods in Immunology and Immunochemistry*, Vol. III (C. A. Williams and M. W. Chase, eds.), pp. 237–273, Academic Press, New York.

Witte, K. H., 1971, Micro-color test for assay of transmissible gastroenteretis virus-neutralizing antibodies, *Arch. Gesamte Virusforsch.* **33**:171.

Witter R., Frank, H., Moennig, V., Hunsmann, G., Lange, J., and Schäfer, W., 1973, Properties of mouse leukemia viruses. IV. Hemagglutination assay and characterization of hemagglutinating surface components, *Virology* **54**:330.

Wolf, P., and Schmelzer, K., 1973, Polyethylene glycol as a means for improving titers of antisera against plant viruses, *Acta Phytopathol. Acad. Sci. Hung.* **8**:351.

Wolff, K. L., Trent, D. W., Karabatsos, N., and Hudson, B. W., 1977, Use of glutaraldehyde-fixed goose erythrocytes in arbovirus serology, *J. Clin. Microbiol.* **6**:55.

Wooley, R. E., Brown, J., Gratzek, J. B., Kleven, S. H., and Scott, T. A., 1974, Microculture system for detection of Newcastle disease virus antibodies, *Appl. Microbiol.* **27**:890.

Wrigley, N. G., Laver, W. G., and Downie, J. C., 1977, Binding of antibodies to isolated haemagglutinin and neuraminidase molecules of influenza virus observed in the electron microscope, *J. Mol. Biol.* **109**:405.

Yanagida, M., 1972, Identification of some antigenic precursors of bacteriophage T4, *J. Mol. Biol.* **65**:501.

Yanagida, M., and Ahmad-Zadeh, C., 1970, Determination of gene product positions in bacteriophage T4 by specific antibody association, *J. Mol. Biol.* **51**:411.

Yoshino, K., and Taniguchi, S., 1965, Studies on the neutralization of herpes simplex virus. III. Mechanism of the antibody-potentiating action of complement, *Virology* **26**:61.

Yung, L. L. L., Loh, W., and Ter Meulen, V., 1977, Solid phase indirect radioimmunoassay: Standardization and applications in viral serology, *Med. Microbiol. Immunol.* **163**:111.

CHAPTER 5

Chemical Modification of Viruses

H. Fraenkel-Conrat

Department of Molecular Biology and Virus Laboratory
University of California
Berkeley, California 94720

1. INTRODUCTION

Viruses were first subjected to chemical modification at a time when this was an approach generally used to characterize biologically active substances and when the nature and the complexity of viruses were not yet understood. After it had been established that viruses were composed of proteins, nucleic acids, and often also lipids, and that they had definite and different architectures and topographies, it became evident that the results of many modification reactions would be difficult to interpret, and such techniques were used less frequently with entire viruses. However, with focus on the results of modification of a specific virus component within the particle, such as the protein or the nucleic acid of the simple viruses, and disregarding "side reactions" of the other component(s), such reactions continued to be of some usefulness. This chapter will summarize the methods, purposes, and conclusions arrived at by chemical (and a few enzymatic) modification reactions of viruses. Photochemical modifications will be dealt with in the following two chapters. We will not discuss the much larger topic of reactions of the separated components or that of agents that bind by other than covalent linkage.

The rationale for most virus modification studies, particularly in

the early days of experimental virology, were either to inactivate them, frequently for the purpose of then using such inactivated viruses to immunize animals or man, or to produce mutants. More recently, studies of the chemical reactivity of viruses have frequently been aimed at the localization of various proteins and other components in determining the fine structure and mode of assembly of virus particles. Whatever the purpose, virus modifications must obviously be performed under conditions where the viruses are stable. Thus all reactions to be discussed are, unless otherwise specified, carried out in at least 90% aqueous media of pH 6–7.5 at 0–37° C, and usually at low reagent concentrations.

This chapter first will deal with the reagents that have been used to modify viruses, and then will focus on the purposes of carrying out virus modifications.

2. REAGENTS USED FOR VIRUS MODIFICATION

2.1. Acylating Agents

Early experiments with typical acylating agents (e.g., acid anhydrides, ketenes, toluene sulfonylchloride, carbobenzoxychloride, and phenylisocyanate; Table 1, 1–5)* showed that these compounds usually caused virus inactivation and often solubility changes; such results were interpreted as evidence for the protein nature of viruses. Although we are now inclined to attribute most virus-inactivating reactions to nucleic acid modifications, this is probably least the case with acylating agents since acylation of nucleic acids does not occur readily and reverses easily. Thus the search for the production of mutants by acylating agents has generally given negative results. Acylation reactions, however, have been successfully used for the structural characterization of virus particles. The action of monofunctional reagents has given data concerning the steric availability of specific groups in viral coat proteins of known amino acid sequence. Thus the finding that in TMV only one of the four tyrosines, residue 139 near the *C* terminus, is acylated by acetic anhydride, as well as one of the two amino groups (residue 68, not 53), has supplied data of importance in establishing the conformation of that protein in the virus particle (Fraenkel-Conrat and Colloms, 1967; Durham and Butler, 1975).

* All numbers in parentheses after the names of reagents refer to the chemical formulas presented in Fig. 1.

Acylating agents have also been used to determine which of several coat or envelope proteins of the more complex viruses are more available for reaction, and thus more external. This type of study does not require a knowledge of the amino acid sequences of the proteins involved. Thus it was established that in the polio and rhino virions VP1 is most reactive, and that VP4 is quite unreactive to acetic anhydride (Lonberg-Holm and Butterworth, 1976). The conclusions concerning the different reactivities in terms of locations of these proteins were borne out by iodination studies to be discussed in Section 2.3. In contrast to the picornaviridae, all proteins and lipids in retroviridae became acetylated to similar extents by acetic anhydride, suggesting a looser structure for such enveloped viruses (Montelaro and Rueckert, 1975). One may surmise that the hydrophobic nature of both the viral envelope and the reagent plays a role in the outcome of these experiments.

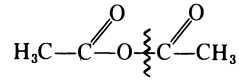
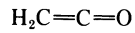
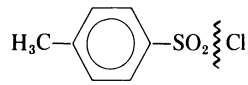
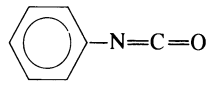
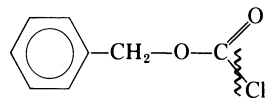
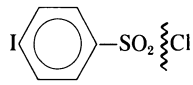
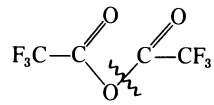
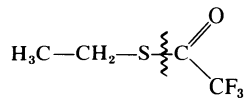
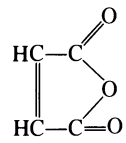
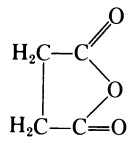
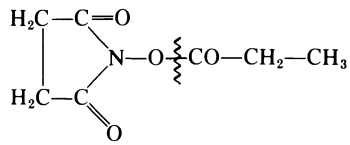
Another important use of these, as of other types of modifications, is to ascertain by means of bifunctional reagents the relative position and proximity of several different proteins occurring in complex virions. Such cross-linking through acylation is achieved by diisocyanates, bisanhydrides, tartryldiazides, etc. These reactions will be discussed in Section 3.1.2.

As far as reaction specificity is concerned, thiol and imidazole groups are the most readily acylated, but are usually also the most labile, so that brief exposure to alkali may hydrolyze such acyl bonds. The same is true to a lesser extent with the phenolic groups, which react similarly to the amino groups (Fraenkel-Conrat and Colloms, 1967). The latter groups, however, because of their usually external location, represent the main acceptors of stably bound acyl groups. Yet there are several well-established exceptions to this ranking. Thus, in TMV protein, two of four tyrosines are readily acetylated, while, in the virus rod, as previously noted, only the tyrosine near the *C* terminus reacts; in both instances, the rates of acetylation of available tyrosines and lysines are similar. Of the two amino groups, that in position 53 is much less reactive than that in position 68 (Fraenkel-Conrat and Colloms, 1967; Perham and Richards, 1968; King and Perham, 1971; Perham, 1973). *p*-Iodobenzenesulfonyl chloride (pipsyl chloride) (6) reacts only with tyrosine residue 139 (Perham, 1973).

Trifluoroacetic anhydride and *S*-ethyltrifluorothioacetate (7, 8) have the advantage of reversibility over acetylation of amino groups, and the latter shows considerable amino group specificity. That reagent has given the same results as acetic anhydride in regard to the role of

TABLE 1

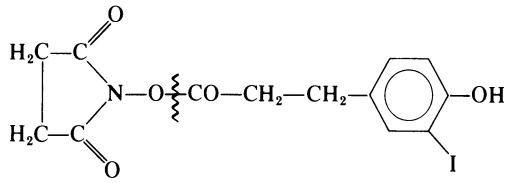
Chemicals Used for Virus Modifications[a]

1.	Acetic anhydride	
2.	Ketene	
3.	Toluene sulfonylchloride	
4.	Phenylisocyanate	
5.	Carbobenzoxychloride	
6.	Pipsyl chloride	
7.	Trifluoroacetic anhydride	
8.	*S*-Ethyltrifluoroacetate	
9.	Maleic anhydride	
10.	Succinic anhydride	
11.	*N*-Succinimidylpropionate	

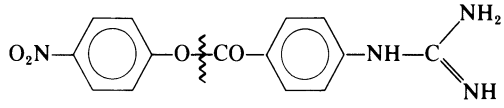
(*Continued*)

TABLE 1. (*Continued*)

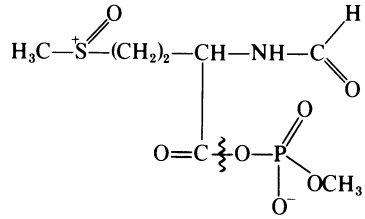
12. *N*-Succinimidyl-3(4-hydroxy-5-iodophenylpropionate) (Bolton-Hunter reagent)



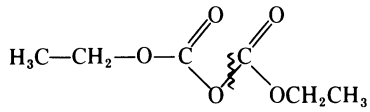
13. *p*-Nitrophenyl-*p'*-guanidino-benzoate



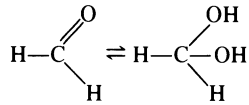
14. Formylmethionylsulfone methyl-phosphate



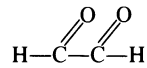
15. Ethoxyformic anhydride (or diethylpyrocarbonate)



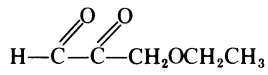
16. Formaldehyde



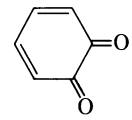
17. Glyoxal



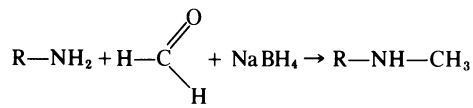
18. Kethoxal



19. *o*-Quinones



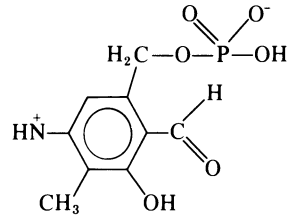
20. Reductive alkylation



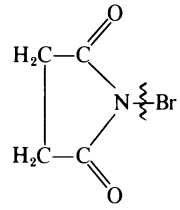
(*Continued*)

TABLE 1. (*Continued*)

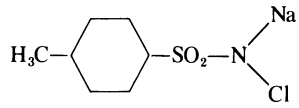
21. Pyridoxal phosphate



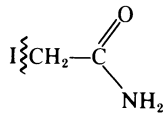
22. *N*-Bromosuccinimide



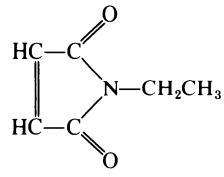
23. Chloramine T



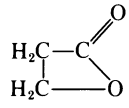
24. Iodoacetamide



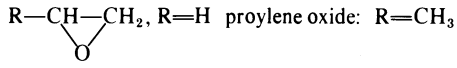
25. *N*-Ethylmaleimide



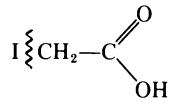
26. β-Propiolactone



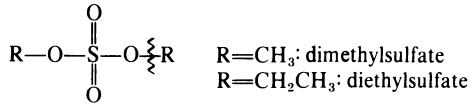
27. Ethylene oxide



28. Iodoacetate

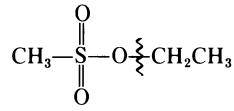
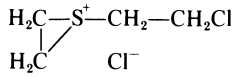
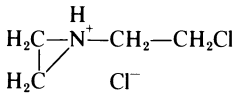
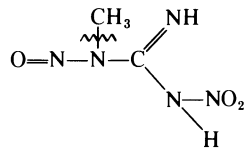
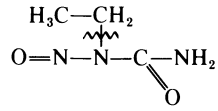
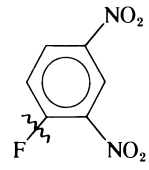
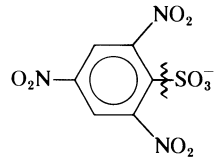
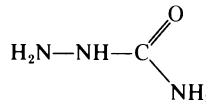


29. Alkylsufate



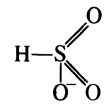
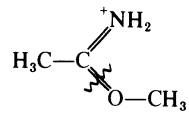
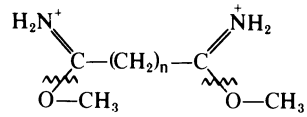
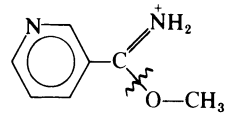
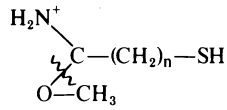
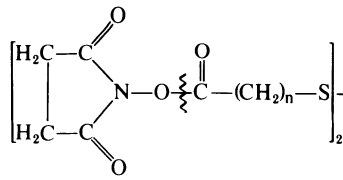
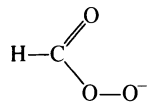
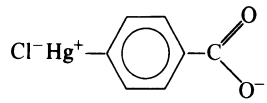
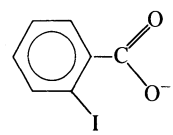
(*Continued*)

TABLE 1. (*Continued*)

30.	Ethylmethane sulfonate	
31.	*S*-Mustard	
32.	*N*-Mustard	
33.	*N*-Methyl-*N*-nitroso-*N'*nitro-guanidine	
34.	*N*-Ethyl-*N*-nitrosourea	
35.	2,4-Dinitrofluorobenzene (DNFB)	
36.	2,4,6-Trinitrobenzene sulfonic acid (TNBS)	
37.	Hydroxylamine	$H_2N—OH$
38.	Hydrazine	$H_2N—NH_2$
39.	Semicarbazide	
40.	*O*-Methoxyamine	$H_2N—O—CH_3$

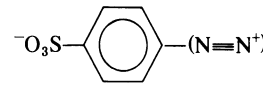
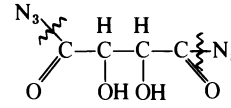
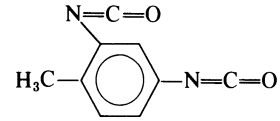
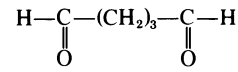
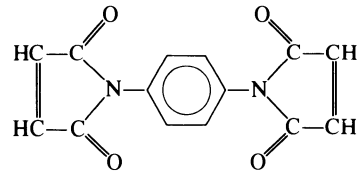
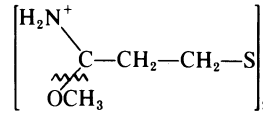
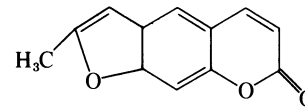
(*Continued*)

TABLE 1. (*Continued*)

41.	Bisulfite		
42.	Methylacetimidate		
43.	Dimethyladipimidate		n=4
44.	Dimethylsuberimidate	Same	n=6
45.	Methylpicolinimidate		
46.	Methyl-3-mercaptopropionimidate		n=2
47.	Methyl-4-mercaptobutyrimidate	Same	n=3
48.	Dithiobissuccinimidyl propionate		n=2
49.	Dithiobissuccinimidyl butyrate	Same	n=3
50.	Performic acid		
51.	Methyl mercuric salts	H_3C-Hg^+ NO_3^- or Cl^-	
52.	*p*-Chloromercuribenzoate		
53.	*o*-Iodosobenzoate		

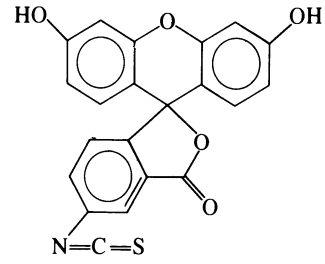
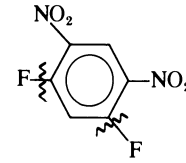
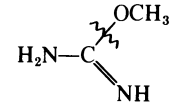
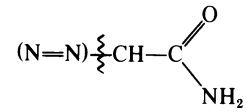
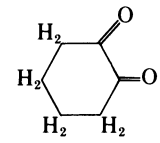
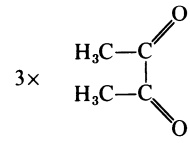
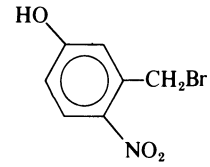
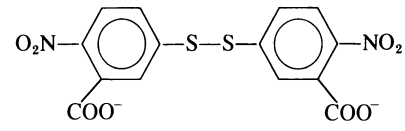
(*Continued*)

TABLE 1. (*Continued*)

54.	Diazosulfanilic acid	
55.	Water-soluble carbodiimide (an example)	$H_3C—CH_2—N=C=N—(CH_2)_3—N(CH_3)_2$
56.	Mercaptoethanol	$HO—CH_2—CH_2—SH$
57.	Dithiothreitol	$HSCH_2—CHOH—CHOH—CH_2SH$
58.	Tartryldiazide	
59.	Toluene-2,4-diisocyanate	
60.	Hexamethylenediisocyanate	$O=C=N—(CH_2)_6—N=C=O$
61.	Glutaraldyhyde	
62.	*N,N'-p*-Phenylenedimaleimides	
63.	*N,N'*-Alkylenedimaleimides	Same $—(CH_2)_n—$ Same n=1—5
64.	Dimethyl-3,3'-dithiobispropionimidate	
65.	Trioxsalen (psoralene derivative)	

(*Continued*)

TABLE 1. (*Continued*)

66. Fluorescein isothiocyanate	
67. 1,5-Difluoro-2,4-dinitrobenzene	
68. *O*-Methylisourea	
69. Diazoacetamide	
70. Cyclohexanedione	
71. Diacetyltrimer	
72. 2-Hydroxy-5-nitrobenzyl bromide	
73. Tetranitromethane	
74. 5,5′-Dithiobis-(2-nitrobenzoic acid)	

[a] The wavy line crossing bonds is to indicate which part of the molecule is released in the binding of the reagent to a viral component.

the two amino groups of TMV coat protein in virus assembly (Perham and Richards, 1968). The action of maleic and succinic anhydride (9, 10) on viruses has also been studied (King and Perham, 1971; Rushizky and Mozejko, 1973; Singh and Terzaghi, 1974; Sehgal and Hsu, 1977). A particular advantage of maleic anhydride is the reversibility of the reaction in weak acid (Butler *et al.*, 1969). At high levels of acylation, these reagents cause extensive degradation, but levels can be found where they acylate only one to three groups; then they show the same selectivity for available amino groups as acetic anhydride. However, acylation of sterically available tyrosine, serine, and threonine groups may also occasionally occur (King and Perham, 1971).

A new type of acylating agents frequently used with radioactive substituents on the acyl group are the succinimidyl compounds, such as succinimidylpropionate. These compounds react with great affinity with available groups, usually the amino groups (11, 12) (see Wetz and Habermehl, 1979).

Among rather untypical and specialized acylating agents that have been used with viruses is the protease inhibitor, *p*-nitrophenyl-*p'*-guanidinobenzoate (NPGB) (13) (Chase and Shaw, 1969). The conclusion concerning its inactivating action on enveloped viruses, as determined for Sindbis virus, is that it is unable to attack the viral proteins but is able to penetrate the lipid bilayer, and it then acylates and inactivates the viral RNA (Bracha *et al.*, 1977*a*,*b*). An acylating agent that is specifically designed to be unable to pass through membranes is [^{35}S]-formylmethionyl sulfone methylphosphate (14); it acylates at pH 10 primarily the ϵ-amino groups, and only those at the surface of Semliki Forest virus (Gahmberg *et al.*, 1972).

Another acylating agent that is of interest, even though it is as nonspecific as the above agent was believed to be specific, is ethoxyformic anhydride (diethylpyrocarbonate) (15). This chemical is highly reactive, and its virus-inactivating action may also be due to its reaction with the nucleic acid, particularly if the acid is single stranded (Ehrenfeld, 1974). TMV virions are not readily inactivated by ethoxyformic anhydride (Oxelfelt and Arstrand, 1970).

2.2. Aldehydes, Ketones, and Reductive Alkylation

Formaldehyde (16) is a classical protein reagent, and its affinity for the amino groups has long been used as a means of titrating these. In a series of papers by the author and various co-workers, it was

shown that, besides the reversible addition reaction of formaldehyde to the amino groups, there occurred slower and more stable cross-linking reactions of the resultant amino-methylols through condensation with many other protein side chains, yielding methylene bridges (e.g., —NH—CH_2—NH—CO—; —NH—CH_2—(C_6H_3OH)—) (Fraenkel-Conrat and Olcott, 1946; Fraenkel-Conrat and Olcott, 1948*a*,*b*). The study of the effect of formaldehyde on TMV actually initiated the author's collaboration with W. M. Stanley's laboratory at Princeton and led to his subsequent association with the U. C. Berkeley Virus Laboratory.

He later showed that formaldehyde reacts in readily reversible fashion with the available amino groups of nucleic acids, and thus much more with single-stranded than with double-stranded nucleic acids (Fraenkel-Conrat, 1954). Methylene bridges resulting in stable cross-linking were subsequently demonstrated by Feldman (1973) to occur between guanine residues.

It appears probable but not proven that the long-known inactivating effects of formalin on microorganisms and viruses are primarily due to the affinity of this compound for the amino groups of the RNA which are unprotonated at pH 6–7. Cross-linking between such —NH—CH_2OH groups and reactive residues on vicinal protein and polynucleotide chains is also a good possibility and would surely be very destructive to viral function (Heicken and Spicher, 1959). A particularly important aspect of formaldehyde modification is that, because of the smallness of the reagent and its lack of hydrophobic groups, it causes minimal disturbance of protein conformation. Its cross-linking action is confined to sterically close groups and is thus likely to stabilize the original conformation by new primary linkages. Thus the antigenic properties of formaldehyde-treated proteins or viruses appear not to differ greatly from those of the original material. It is for this reason that formaldehyde treatment has been and remains the preferred method of producing "killed vaccines," which are biological materials the toxicity of which has been abolished without loss or change of their serological properties. The presence of methylene bridges between lysine and tyrosine side chains has actually been demonstrated in formalin-treated bacterial toxins (Blass *et al.*, 1965).

Formalin treatment is what Salk (1955) used in producing the first polio vaccine, a landmark in the history of medicine. Certain small-molecular alkylating agents (β-propiolactone, ethylene oxide, etc.; see section 2.4), as well as UV light have also been used at times to prepare viral vaccines. The question whether "killed" vaccines are to be pre-

ferred over live, attenuated virus strains as means of producing immunity (Salk vs. Sabin in regard to polio) has been argued at length over many years. At present, the ever-increasing evidence for possible contamination of all biological materials with cryptic tumor viruses seems to favor the chemical methods of inactivating viruses for the purposes of vaccine preparation. Theoretically the best method, although unfortunately not often practical, is surely that advocated by Fraenkel-Conrat *et al.* (1959) of irreversibly inactivating the isolated viral RNA by reagents such as formaldehyde or β-propiolactone, and subsequently reconstituting it with unmodified viral coat protein to serologically pristine virions. This was actually accomplished successfully with TMV (Staehelin, 1960). Theoretically even better would appear to be the use of only the viral coat protein as the antigen (see Chapter 4). However, unless the protein can be assembled into viruslike particles, its antigenicity is neither qualitatively nor quantitatively equivalent to that of the original virus, and such nucleic-acid-lacking protein shells are generally not very stable.

While monofunctional aldehydes can only make short (—CH_2—) cross-links, dialdehydes, e.g., glutaraldehyde, have been used to produce intermolecular cross-links (Section 3.1.2) and thus establish relative proximity of protein molecules (Schäfer *et al.*, 1975; Sehgal and Hsu, 1977). In these, as in almost all reactions of viruses, inactivation is more probably attributable to the interaction of the reagent with the nucleic acid rather than with the protein. Glutaraldehyde has proven particularly useful in preparing specimens for electron microscopy. This "tanning" action of aldehydes has obviously been of considerable usefulness for a long time.

Glyoxal (Thomas and Hannoun, 1957; Thomas *et al.*, 1957) (17) and its more stable homologue, Kethoxal (18), have a particular affinity for guanine residues (Staehelin, 1960), where the cross-linking action results in transforming into a five-membered ring the 1- and 2-amino positions of the base, a reaction that occurs much more readily in single-stranded than in double-stranded nucleic acids. While Kethoxal is, on TMV-RNA, more inactivating than glyoxal and formaldehyde in the virus the order of efficiency of these three reagents is inversely related to their molecular weights (Staehlin, 1960). The *o*-quinones (19) resulting from oxidation of plant phenolic compounds were also found to react with viral proteins, but these reactions have not been studied in detail (Pierpoint, 1973).

Reductive alkylation (20) represents a useful extension of the use of aldehydes in protein modification (Means and Feeny, 1968). This is

achieved by reducing the primary aldehyde–amine addition or condensation produce with sodium borohydride. With formaldehyde, this leads to ϵ-*N*-methyl lysine residues with high specificity, a particularly useful modification because it allows the easy introduction of a radioactive label into proteins (either as [^{14}C]formaldehyde or as [^{3}H]borohydride) (Rice and Means, 1971). Such modification has the advantage over other labeling procedures, such as iodination or acylation, of having no marked effect on the physicochemical properties of the modified protein. This reaction has not been used with viruses as much as one might expect.

The corresponding reaction with pyridoxal phosphate (21) has been used to test the availability of certain amino groups to this large, charged, and UV-absorbing aldehyde (Pierpoint, 1974; Pierpoint and Carpenter, 1978). By means of ^{32}P-labeled reagent, high specific activities can be introduced into viruses (Eger and Rifkin, 1977).

A variation on this theme is to generate the aldehyde in the envelope of viruses by treatment with galactose oxidase, and then to label the available site by reduction with [^{3}H]-$NaBH_4$ (Luukkonen *et al.*, 1977).

2.3. Halogenating Agents

Molecular halogens, as well as *N*-bonded halogens such as *N*-bromo-succinimide (22) are quite reactive and usually harmful to viruses, probably largely because of their reaction with the nucleic acids (Young *et al.*, 1977). Thus chlorine, forming H-O-Cl in water, is frequently used as a nonspecific inactivating agent (e.g., Vorob'eva *et al.*, 1978). However, under carefully controlled conditions, iodine and bromine compounds can be used for specific modification purposes.

2.3.1. Iodination

Iodine is possibly the most useful and versatile tool of virus modification, again with particular reference to the proteins of virions. Iodine, usually in the form of I_3 or ICl, rapidly oxidizes available SH groups, and allowance must always be made for this reaction in —SH-containing proteins unless the —SH group is in some way protected. The first step in the reaction of —SH with iodine is the formation of a sulfenyl iodide group (—SI), which rapidly decomposes to —S—OH and then becomes oxidized further. It was thus a surprising finding that the

single —SH group of the TMV protein in the virion yields a stable sulfenyliodide group, giving the virus a yellow color (Fraenkel-Conrat, 1955). This represented an early illustration of the power of the quaternary structure of a protein in affecting the reactivity of specific groups through creation of particular microenvironments. Recently, the poliovirus was found to bind much more iodine than was found, on dissociation, to be bound by its components, and it was suggested that sulfenyliodide formation, stable only in the virion, might be the explanation for this observation (Lonberg-Holm and Butterworth, 1976).

The iodination of tyrosine and at times histidine residues in proteins represents the main objective in the use of iodine for a variety of purposes and under different conditions. Different tyrosine residues may show different reactivities in the virion as contrasted to its isolated native or denatured proteins, and these differences again give information about the conformation of such proteins under different conditions (Fraenkel-Conrat and Sherwood, 1967). Iodine treatment of crystals of TMV protein disks (33 molecules) have shown that, here also, the tyrosines near the *C* terminus and to a lesser extent the *N* terminus are the most reactive (Graham and Butler, 1978). Even the relative reactivity of the two orthopositions of a given tyrosine residue may differ under different conditions, resulting in varying proportions of mono- and diiodotyrosine being formed.

A far more trivial use of iodine in virology lies in its availability in high-specific-activity radioactive forms (^{125}I, ^{131}I) which makes it a convenient tool for labeling and detecting very small amounts of viral material.

The use of iodination that has found greatest interest in recent years is as a tool in determining the location of specific proteins in the particles of many different viruses. The grounds for this application is that iodine can be generated not only by oxidation of iodide with small molecules such as H_2O_2 or chloramine T (23) (Hunter and Ludwig, 1962), but also enzymatically by peroxidases in conjuction with very low peroxide concentrations (Marchalonis, 1969; Phillips, and Morrison, 1970). The use of lactoperoxidase appears most effective. Its molecular weight of 78,000 restricts its action to iodination only of proteins located at or near the surface of a virus particle. Thus the approximate position of virus proteins within a particle can be deduced from the extent to which they become enzymatically iodinated, as compared to their reactivity after degradation of the particle, or with chemically produced iodine. Such methodology has been successfully applied to members of most virus families (see Table 2). Through coupling of the lactoperoxidase to sepharose, the generation of iodine can be rendered

TABLE 2

Tests for Surface or External Location of Viral Proteins

Method, Reagent	Virus	References
Acetic anhydride	Poliomyelitis	Lonberg-Holm and Butterworth (1976)
Fluorescein isothiocyanate (66)	Vaccinia	Sarov and Joklik (1972)
Formylmethionylsulfone methylphosphate (14)	Semliki Forest	Gahmberg *et al.* (1972) Garoff and Simmons (1974)
Iodine—lactoperoxidase	Phage ϕ6	Van Etten *et al.* (1976) Sehgal and Hsu (1977)
	PM2	Brewer and Singer (1974)
	Southern bean mosaic	Van Etten *et al.* (1976)
	Adeno	Everitt *et al.* (1975)
	Adeno-associated	Lubeck and Johnson (1977)
	Bovine entero	Carthew and Martin (1974)
	Influenza	Stanley and Haslam (1971)
	Mengo	Lund *et al.* (1977)
	Mouse mammary tumor	Witte *et al.* (1973)
	Poliomyelitis	Lonberg-Holm and Butterworth (1976) Beneke *et al.* (1977)
	Sindbis	Sefton *et al.* (1973)
	Vesicular stomatitis	McSharry (1977)
	Vaccinia	Sarov and Joklik (1972) Katz and Margalith (1973)
Iodine—solid state	Adeno-associated	Lubeck and Johnson (1977) David and Reisfeld (1974)
Iodine—chloroglycoluril	Sendai	Markwell and Fox (1978)
	Newcastle disease	Markwell and Fox (1978)
Iodoacetate (28)	Poliomyelitis	Lonberg-Holm and Butterworth (1976)
Phospholipase C	Vesicular stomatitis	Cartwright *et al.* (1969)
	Influenza	Rothman *et al.* (1976)
Trypsin	Vaccinia	Sarov (1972)
	PM2	Brewer and Singer (1974)
	Vesicular stomatitis	Cartwright *et al.* (1969)
Chymotrypsin	Bovine entero	Carthew and Martin (1974)
	Foot and mouth disease	Cavanagh *et al.* (1977)
Transglutaminase	PM2	Brewer and Singer (1974)
Bromelain	PM2	Hinnen *et al.* (1974)
Pronase	Vesicular stomatitis	McSharry *et al.* (1971)
	Corona	Sturman (1977)
Galactose oxidase + $[^3H]$-$NaBH_4$	Semliki Forest	Luukkonen *et al.* (1977)

yet more superficial, as was done in showing that all three proteins of adeno-associated virus are at the surface (David and Reisfield, 1974; Lubeck and Johnson, 1977).

To achieve high radioactive iodine labeling without addition of iodine and oxidants to sensitive viruses, the use of the Bolton-Hunter reagent in particular can be recommended. Another recently advocated method for gentle labeling of surface proteins utilizes a water-insoluble film of an oxidant, chloroglycoluril, in conjunction with carrier-free Na ^{125}I (Markwell and Fox, 1978).

2.3.2. Bromination

The reaction of bromine-water with viruses appear not to have been studied in detail. It readily reacts with all nucleic acid bases except adenine in RNA and DNA (Brammer, 1963). The primary product with the pyrimidines is the 5,6 addition of BrOH; the reaction with guanosine is more complex. Thus any inactivating effects of brominating agents on viruses are to be attributed to these reactions, although the primary reaction products do not appear to account for the mutagenicity of these reactions (Means and Fraenkel-Conrat, 1971).

N-Bromosuccinimide (22) has been advocated as a specific peptide-chain-cleaving agent in attacking the carbonyl bond of tryptophane. However, it was always recognized that, under the rough conditions necessary to achieve this, other groups would also be oxidized or brominated, with particular reference to the tyrosine residues. Under carefully controlled conditions near neutrality, reaction can be confined to the first step of the tryptophan modification, hydroxybromination of the double bond of the pyrole ring. That the reaction of *N*-bromosuccinimide with proteins is again quite conformation dependent is illustrated by the report that only two of the three tryptophans of TMV protein become oxidized, and that, in the virion and in protein rod aggregates, tryptophan does not react at all (Fairhead *et al.*, 1969). More recently, it was found that actually tyrosine 139 of the TMV protein is the first site of interaction, becoming dibrominated (Okada *et al.*, 1972); it is this same tyrosine that is the most readily iodinated and acetylated (Fraenkel-Conrat and Sherwood, 1967; Fraenkel-Conrat and Colloms, 1967). In the light of these findings, it is particularly surprising that the tryptophan near this tyrosine and the exposed *C* terminus, residue 152, is the only one to be completely resistant to *N*-bromo-succinimide oxidation in the virus protein. Bromination of the reactive

tyrosine did not prevent TMV protein from reconstituting stable and infective virus (Ohno *et al.*, 1972).

2.4. Alkylating and Arylating Agents

The relative reactivity of protein groups to alkylation is generally quite pH dependent; at neutrality, it is thiol>imidazole>amino>phenol groups. However, in acid, methionine is the most reactive, and in alkali, the amino groups become extensively alkylated. Apart from these general rules, the actual reactivities in each protein are much more a consequence of its specific conformation than of any such order of affinities. The —SH groups in viral proteins are in general conformationally masked, but when, even after dissociation of the virus particle, a protein does not readily react with a reagent such as iodoacetamide (24) or *N*-ethylmaleimide (25) which also acts as an alkylating agent, this is taken as evidence that the protein lacks —SH groups.

Alkylating agents had been used with viruses for a long time before it was realized that these agents had a great affinity for nucleic acids and that their virus-inactivating effects were largely due to these reactions. Agents such as β-propiolactone (26) (Brown *et al.*, 1974; Brusick, 1977), ethylene or propylene oxide (27) (Hoff-Jørgensen and Lund, 1972), and iodoacetate or its amide (28, 24) (Singh and Terzaghi, 1974; Lonberg-Holm and Butterworth, 1976) have been used frequently in recent years to achieve virus genome inactivation. The main target for these reagents, as well as for dimethylsulfate (29), is the 7-position of the guanosine residues (Lawley *et al.*, 1969). The lability of the glycosidic bond of this and other alkylated purines which leads to spontaneous depurination in DNA appears to contribute to the virus inactivating action of such alkylations (Shooter *et al.*, 1974*a*,*b*; Shooter, 1975; Karska-Wysocki *et al.*, 1976).

Many comparative studies of the action of alkylating agents such as β-propiolactone, dialkyl sulfates, alkyl alkanesulfonates (30), and *S*- and *N*-mustards (31, 32) on bacteriophages have been reported, with particular concern for their inactivating and mutagenic action (Corbett *et al.*, 1970; Ray *et al.*, 1972). There are no detailed reports, to our knowledge, concerning the extensive modification of the phage proteins by these various alkylating agents. There are, however, several reports concerning their effects on the biological capabilities of treated phages. Thus it is reported that bifunctional alkylating agents (e.g., *S*-mustards and *N*-mustards), while not interfering with adsorption of the phage,

frequently prevent the transfer of the DNA into the bacterium (Lawley *et al.*, 1969; Shooter *et al.*, 1971–1975; Karska-Wysocki *et al.*, 1976). This probably signifies a cross-linking between phage coat proteins and DNA, and as such is of interest from the phage structural point of view.

A particularly interesting result of methylnitrosonitroguanidine (MNNG) (33) treatment of TMV was that this reagent alkylated the RNA and was highly mutagenic when acting on the virus, but not when acting on the viral RNA (Singer *et al.*, 1968; Singer and Fraenkel-Conrat, 1969*a*,*b*). The interpretation that this was due to a proportionately higher alkylation of C than G in the virus is now recognized not to explain the observed and confirmed high mutagenesis of methyl- and ethylnitrosoguanidines (MNNG, ENNG) on TMV. Although 3-MeC has been firmly established to represent a mutagenic base (Ludlum and Wilhelm, 1968; Singer and Fraenkel-Conrat, 1970; Singer *et a.*, 1979; Kröger and Singer, 1979), these reagents, like all *N*-alkyl-*N*-nitroso compounds, have since been found to alkylate all oxygens of nucleic acids much more than the nitrogens (Singer, 1976; Singer *et al.*, 1978). Thus methylnitrosonitroguanidine yields more of the also mutagenic *O*-methyl U and C derivatives than of 3-MeC, and produces these in similar proportions in the TMV virion as in the isolated RNA. The particularly high mutagenicity of alkyl nitrosonitroguanidines when acting on the virion, as contrasted to the RNA, thus remains unexplained, particularly in view of the fact that the other nitroso-alkylating agents show low mutagenicity with TMV and its RNA, resembling the simple alkyl sulfates and alkyl alkanesulfonates in this regard. That the mutagenicity of MNNG is a very complex phenomenon is also indicated by a recent study using phage λ (Yamamoto and Kondo, 1978).

It has become evident in recent years that all *N*-nitrosoalkylating agents, whether formed in the body or produced environmentally, are highly carcinogenic, either directly or after metabolic activation. The nitroso-alkyl ureas (34) are directly carcinogenic; thus their action on viruses and their nucleic acids has been intensely studied in recent years. As stated above, these agents also alkylate with great preference all oxygens in nucleic acids, including those on ribose and phosphate, the ethyl derivative being less reactive but more oxygen specific and carcinogenic than methylnitrosourea (Singer, 1976; Smith, 1976; Singer *et al.*, 1978). Their effects on proteins, including viral proteins, remain to be determined. That the mutagenizing action of ethylnitrosourea on TMV and its RNA is similarly low as that of the simple alkylating agents, quite in contrast to the action of MNNG and ENNG on TMV, discussed above, also remains a puzzle to be resolved in the future. Most of the actions of alkylnitrosourea on nucleic acid bases are mutagenic,

and all compounds of this class are definitely highly carcinogenic. The many detailed studies of the correlations between the chemical action of the alkylnitroso compounds on nucleic acids and their lethality, mutagenicity, and carcinogenicity is beyond the scope of this chapter dealing with virus modifications. The reader is referred to several excellent reviews on this subject (Singer, 1975, 1977).

It has been recognized in recent years that carcinogenic polycyclic hydrocarbons (e.g., 7-bromomethylbenz[a]anthracenes), while not becoming covalently bound by viruses, also yield alkylating agents in the course of metabolic "detoxification," namely, epoxides. A few studies deal with the effect of these "activated" compounds on viruses (e.g., Lotlikar *et al.*, 1972; Dipple and Shooter, 1974; Singer *et al.*, 1980); many more studies use only viral nucleic acids, which in any case are the more reactive and more interesting target compounds. We refer the reader to a recent review of this topic (Grunberger and Weinstein, 1979).

Certain large arylating compounds such as 5-fluoro-2,4-dinitrobenzene (FDNB) (35) and 2,4,6-trinitrobenzene sulfonate (TNBS) (36) have also been used occasionally to ascertain the relative availability of the amino groups of viral proteins (e.g., Scheele and Lauffer, 1969; Perham, 1973; Singh and Terzaghi, 1974; Kaper, 1976). None of these reagents are any more amino-specific than typical alkylating agents, but their color and/or UV absorbance represents an analytical advantage over simple alkylating agents.

2.5. Pyrimidine Modifications (Amines, Bisulfite, etc.)

Various modifications of viruses result secondarily from the primary addition of nucleophilic compounds such as hydroxylamine, hydrazine, semicarbazide, etc. (37–39) to the C5, C6 double bond of the pyrimidines, reactions which are generally quite reversible.* These additions lead to activation of the exocyclic amino group of cytosine, which then tends to become replaced by a substituted nitrogen, or deaminated. The mutagenicity of hydroxylamine and *O*-methoxyamine (40) is due to such replacement reactions and consequent tautomeric shift (Maes and Mesquita, 1970; Fraenkel-Conrat and Singer, 1972; Budowsky *et al.*, 1974; Newlin and Bussell, 1975; Khromov *et al.*, 1977). The lability of the double-bond addition products may account for phenomena such as the ability of MS2 phage to spontaneously

* The addition of water to the double bond under the influence of UV light is discussed in Chapter 6.

recover some of its lost infectivity (Budowsky and Pashneva, 1971; Budowsky *et al.*, 1974).

Bisulfite (41) deaminates cytosine to uracil by the same mechanism, but, in the presence of amino compounds, it greatly potentiates transamination and mutagenicity. Partial recovery of infectivity can also be observed, particularly with semicarbazide (Hayatsu, 1977; Hayatsu and Shiragami, 1979). If protein amino groups are in the vicinity, they can replace the amino group of cytosine; primary nucleic acid–protein cross-linking is the consequence, a reaction that is also potentiated by bisulfite (Krivisky *et al.*, 1973; Turchinsky *et al.*, 1974; Khromov *et al.*, 1977; Sklyadneva *et al.*, 1978). These cross-linking reactions (Tikchonenko *et al.*, 1971, 1973; Andronikov *et al.*, 1974) with particular reference to bacteriophages, of both RNA and single- and double-stranded DNA type, have been discussed by Tikchonenko in Volume 5 of this series. Poliovirus, on a single lethal hit by hydroxylamine, loses all early intracellular functions (Borgert *et al.*, 1971). It must be noted, however, that the inactivation of ϕX174 and that of its naked DNA proceed at similar rates, which suggests that there is little or no chance of such cross-linking in this phage (Krivisky *et al.*, 1973). It also appears of interest to note that the intraphage double-stranded DNA of S_D is similarly available to these reagents as single-stranded nucleic acids, while that isolated DNA (Sklyadneva *et al.*, 1970; Tikchonenko *et al.*, 1971) and T2 DNA (Hayatsu, 1977) are very unreactive. This is in agreement with other indications that the tight packaging of DNA leads to much loss of complementary base interaction.

Several authors have noted that different families of enveloped viruses show great differences in their sensitivity to hydroxylamine (2 M) and its derivatives at pH 7.0 (Franklin and Wecker, 1959; Newlin and Bussell, 1975). It appears likely that the structure of the different envelope components and their reactivity toward these agents accounts for the sensitivity of the orthomyxoviridae and the relative insensitivity of the paramyxoviridae.

Another indication that reagents of this type, under certain circumstances, can affect proteins rather than nucleic acids was reported by Kudo *et al.* (1978).

2.6. Imidoesters

Compounds of the type of methylacetimidates (42) have come into increasing use because of their specificity for substituting the amino groups of proteins (Hunter and Ludwig, 1962; Davies and Stark, 1970).

(Reaction with the amino groups of nucleic acids does not seem to have been studied, but appears possible). The main drawback of these reagents is that they usually require somewhat basic media, e.g., pH 8.5. The presence of $MgCl_2$ appears favorable in the modification of viruses labile at that pH, such as the bromoviridae (Bancroft and Smith, 1975). Of particular advantage are the bifunctional members of this group, e.g., dimethyladipimidate and dimethylsuberimidate (43, 44), because of their ability to form cross-links across definite distances (Davies and Stark, 1970; Lomant and Fairbanks, 1976) (see Section 3.1.2). However, the recent finding that these reagents can act in polymeric fashion throws some doubt on the quantitative aspects of such data (Siezen, 1979).

Methylpicolinimidate (45) has been used on various instances in the hope that the metal-chelating property of the derivative would prove useful for X-ray diffraction studies (see Section 3.2). Its UV absorbance makes it easy to determine the extent of reaction, which appears to be rather specifically only with the amino groups, probably for reasons discussed in Section 2.1. Several mutants of TMV with additional lysine residues have been modified in that manner and for this purpose. These residues were found to show different reactivity depending on their location in the molecule (Perham and Richards, 1968; Perham, 1973).

Another attempt to facilitate X-ray diffraction studies that makes use of an imido ester is the use of methyl-3-mercaptopropionimidate (46), which gives the single reactive lysine of TMV a mercury-binding capability (Perham and Thomas, 1971). This reagent or its butyl analogue (47) (Traut *et al.*, 1973) can also be used for the creation of reversible cross-links by oxidizing the —SH group with hydrogen peroxide either before or after the coupling to protein amino groups (Dubovi and Wagner, 1977) (see Section 3.1.2). The same purpose is served by dithiobis-succinimidyl propionate or butyrate (48, 49), acylating agents of the type discussed previously (Lomant and Fairbanks, 1976).

2.7. Nitrous Acid (HNO_2)

Nitrous acid is the classical reagent for amino nitrogen analysis, causing gaseous nitrogen to be formed in acid solution. The amino group thus become replaced by a hydroxyl group. Since the reaction proceeds at a significant rate only below pH 4.5 and also is not particu-

larly specific, it has found little use for virus protein modification. Its action on nucleic acid, in contrast, has made it the chemical mutagen par excellence, since it transforms cytosine to uracil; deamination of adenine to hypoxathine is also quite straightforward and of biological interest (Schuster and Schramm, 1958; Mundry and Gierer, 1958; Gierer and Mundry, 1958; Boeye, 1959; von Vielmetter and Weider, 1959; Vielmetter and Schuster, 1960; Robinson, 1973). Its action on guanine is more complex, yielding other products besides the expected xanthine, and leading to cross-linking (Shapiro, 1964; Shapiro *et al.*, 1977).

When viruses are treated with nitrous acid, the reactivity of the three amino bases of their nucleic acid differs at times in characteristic manner from their intrinsic reactivity, which must be interpreted as a consequence of intraviral nucleic acid conformation (e.g., Schuster and Wilhelm, 1963). The inactivation of different viruses was found to be both much faster (Sehgal, 1973) and much slower (Sehgal and Krause, 1968) than that of their isolated nucleic acid under the same conditions. When it is slower, this is attributed to protection of the RNA by the coat. When it is faster, there is usually evidence for protein–nucleic acid covalent linkage. This is probably also the explanation for progressive inactivation of nitrous-acid-treated ϕX174, which is not observed on storage of the DNA (Lytle and Ginoza, 1970). Cross-linking of intraphage double-stranded DNA has also been observed and explained (Shapiro, 1964; Shapiro *et al.*, 1977). It appears probable that these various side reactions are due to formation of the intermediate diazo compounds which complicate the nitrous acid reaction more than is usually assumed.

2.8. Sulfhydryl Reagents

Thiol groups, being the most reactive amino acid sidechain groups, have been mentioned in almost every one of the preceding sections. A coordinated critical evaluation of means of modifying these groups appears all the more needed. Since thiol groups are susceptible even to atmospheric oxygen, they are, in most proteins, at least partly masked by conformational restriction. When the —SH groups are sterically available, they yield with acylating agents usually labile acyl derivatives, and with alkylating agents quite stable products. They become oxidized by agents such as halogens, performic acid (50), and hydrogen peroxide. Thus when selective removal or substitution of groups other

than SH are the objective, these must usually first be blocked, or special reagents, conditions, or conformational situations must be exploited.

The substitution by iodine of the single —SH group of TMV coat protein in the virion (Fraenkel-Conrat, 1955) represents an example of conformational control. Control by means of specificity often utilizes the affinity of thiols for mercury. Mercuric chloride ($HgCl_2$), methyl mercuric salts (51), and *p*-chloromercuribenzoate (52) are convenient for such purposes (Fraenkel-Conrat, 1958). As expected, the first two can react with —SH groups which may not be accessible to the larger reagent in the intact virus. Mercury compounds are often useful as a means of disrupting virions in controlled and monitorable fashion (Dorne and Hirth, 1971; Kaper and Jenifer, 1968; Philipson, 1964). Other relatively specific —SH reagents, when used under gentle conditions, are *N*-ethylmaleimide (25) and *o*-iodosobenzoate (53). [The latter was recently reported as splitting peptide chains after all tryptophan residues selectivity in 80% acetic acid at ambient temperature (Mahoney and Hermodson, 1979).] Since many enzymes, including the reverse transcriptase of the retroviridae, have essential thiol groups, this type of reagent can be utilized to pinpoint the role of such enzymes in viral infection (Hung, 1973).

2.9. Other Reagents

Diazotized sulfanilic acid (54) and other diazo compounds have occasionally been used, although they are of low specificity. Diazosulfanilic acid tends to react with imidazole and amino groups, but seems to prefer the amino groups of phospholipids in the case of PM2 phage (Hinnen *et al.*, 1974).

Water-soluble carbodiimides (55) couple amines or amino acids to carboxyl groups, groups that are not readily modifiable under conditions where viruses are stable. In TMV, three carboxyls near the *C* terminus were found to accept cystamine under the influence of this reagent (King and Leberman, 1973).

When it is necessary to modify disulfide groups, two approaches can be used. Oxidation by reagents such as performic acid requires rough conditions and is not very specific for disulfide groups; it has, however, proven very useful in sequence analysis.

Complete reduction by great excess of mercaptan, e.g., mercaptoethanol (56) or dithiothreitol (57), proceeds quickly at neutrality and is thus applicable to biological materials. The great autoxidizability of the

many resulting —SH groups, however, represents a serious drawback. A methodology to overcome this by alkylation immediately following the reduction, and preferably in the presence of the reagent thiol, was developed by Fraenkel-Conrat *et al.* (1951). This approach assures quantitative and stable abolishment of all disulfide bonds. Iodoacetate and iodoacetamide have proven most useful for this purpose. The sensitivity of viral infectivity to gentle treatment with reducing agents has also been used to determine the role of available disulfide bonds in the virion's structure (Carver and Seto, 1968).

An enzymatic method has been used for the purpose of modifying glutamine groups. By means of transglutaminase isolated from guinea pig liver, about 1 mole of radioactive glycine ester was introduced into several of the protein chains of PM-2 phage (Brewer and Singer, 1974).

Degradative enzymes have frequently been used to study the availability of certain proteins, glycoproteins, and lipids, and thus obtain evidence about their location relative to the virion's surface. Some of these applications are listed in Table 2.

3. MODIFICATIONS FOR SPECIFIC PURPOSES

3.1. Location of Viral Components

3.1.1. In Regard to Surface of Virion (Table 2)

By far the most useful reaction to ascertain the location of virus proteins in relation to the virion's surface is iodination. The advantage of this modification is the fact that the reactive iodine species can be generated at different levels of the virion through the use of the easily diffusable oxidants such as H_2O_2 or chloramine T, or of enzymes of various molecular weights and dimensions such as lactoperoxidase. By binding the enzyme to a solid support, its action can be made even more surface specific, and the need to separate the iodinated particle and the enzyme can be avoided. Much of our information about the architecture of virions comes from this type of study (Table 2). Other reagents used for this purpose are also listed in that table.

3.1.2. In Regard to Other Components (Table 3)

Cross-linking between intra- and particularly intermolecular protein groups has become a powerful tool in the study of virion structure,

TABLE 3

Relative Location of Virion Proteins by Cross-Linking

Method, reagent	Virus	Reference
1,5-Difluro-2,4-dinitro-benzene (67)	Murine and feline leukemia	Pinter and Fleissner (1979)
Dimethyladipimidate (43)	Cowpea chlorotic mottle	Bancroft and Smith (1975)
	Mengo	Hordern *et al.* (1979)
	Polio	Wetz and Habermehl (1979)
Dimethylsuberimidate (44)	Influenza	Wiley *et al.* (1977)
	Mengo	Hordern *et al.* (1979)
	PM2	Schäfer *et al.* (1975)
	Polio	Wetz and Habermehl (1979)
	Semliki Forest	Garoff (1974)
	Southern bean mosaic	Sehgal and Hsu (1977)
Dimethyl-3,3′-dithiobis-propionimidate (64)	Influenza	Wiley *et al.* (1977)
	Newcastle disease	Nagai *et al.* (1978)
	Semliki Forest	Richardson and Vance (1978)
	Vesicular stomatitis	Mudd and Swanson (1978)
Dithiobissuccinimidyl propionate (48)	Murine and feline leukemia	Pinter and Fleissner (1979)
	Mengo	Hordern *et al.* (1979)
	Semliki Forest	Richardson and Vance (1978)
	Vesicular stomatitis	Dubovi and Wagner (1977)
Formaldehyde (16)	Mengo	Hordern *et al.* (1979)
	Southern bean mosaic	Sehgal and Hsu (1977)
	Vesicular stomatitis	Dubovi and Wagner (1977)
		Brown *et al.* (1974)
		Mudd and Swanson (1978)
Glutaraldehyde (61)	PM2	Schäfer *et al.* (1975)
	Sindbis	Brown *et al.* (1974)
	Vesicular stomatitis	Brown *et al.* (1974)
Hydrogen peroxide	Vesicular stomatitis	Dubovi and Wagner (1977)
	T7	Hartman *et al.* (1979)
Methylmercaptobutyrimidate (47)	Vesicular stomatis	Dubovi and Wagner (1977)
N,N′-p-Phenylenedimale-imide (62)	PM2	Schäfer *et al.* (1975)
Tartryldiazide (58)	Adeno	Everitt *et al.* (1975)
	Vesicular stomatitis	Dubovi and Wagner (1977)
Toluene 2,4-diisocyanate (59)	PM2	Schäfer *et al.* (1975)

function, and assembly. The simplest reagent with such capability is formaldehyde, which can create methylene cross-links. Wider gaps can be bridged by a great number of bifunctional agents, such as tartryldiazide and higher homologues (58); diisocyanates (59, 60); difunctional alkylating agents (*N* or *S*-mustard gas (31, 32); dialdehydes such as glutaraldehyde (61); bismethylimidates (43, 44); phenylene or alkylene

dimaleimides (62, 63). The last-named one is —SH specific. The others will react, as their monomeric equivalents, mainly with available —SH and $—NH_2$ groups.

Of particular advantage are agents that cross-link in reversible manner. Hydrogen peroxide has been used to cross-link —SH groups to disulfide bonds. Other reagents introduce thiol groups or disulfide bonds (64, 48, 49), but all of these cross-links present problems due to the possibility of disulfide exchange. Results obtained with tartryl-type cross-linking agents (58), which are available as several homologues of different molecular length and which are split by gentle periodate treatment, thus appear of greater reliability. Applications of these types of studies are listed in Table 3.

In combination with near-UV irradiation, H_2O_2 has also recently been reported to cross-link T7 DNA and protein (Hartman *et al.*, 1979). Other light-catalyzed inactivating agents which may cause cross-linking are 8-methoxy psoralene and chlorpromazine, both used in conjunction with long-wavelength UV light (Esipova *et al.*, 1978; Hanson, 1979). Various psoralene derivatives are beginning to come into use for intraviral localizations, e.g., Trioxsalen (65) (Shen *et al.*, 1977) and certain other derivatives (Hearst and Thiry, 1977; Hallick *et al.*, 1978). Since these are much more active on viruses containing double-stranded than single-stranded nucleic acids, it appears probable that nucleic acid cross-linking is the predominant reaction. In general, these agents and in particular the four substituted psoralenes are inactivating all kinds of viruses (e.g., EMC, reo, herpes) at low concentrations and cross-link at higher concentrations. The actions of psoralene-type compounds are also discussed in Chapter 6.

3.2. Isomorphic Replacement for X-Ray Diffraction Strains

Since SH groups, because of their affinity for mercury, have proven considerably useful for X-ray diffraction studies of viruses, introduction of additional —SH groups at clearly defined sites is of interest. Methyl-3-mercaptopropionimidate (46) was used with TMV for this purpose (Perham and Thomas, 1971). Maleic anhydride modification of TMV was performed for the same purpose, since it was known that mercuric acetate could be subsequently added to the double bond. Methylpicolinimidate has also been used because of the metal-chelating ability of this group.

3.3. For Inactivation and Mutagenesis

Reagents which have particular affinity for nucleic acid groups are most effective for inactivation and mutagenesis. Reactions involved are alkylation and pyrimidine modification (Sections 2.4 and 2.5). Nitrous acid, because of its action on nucleic acid bases, even though this occurs less frequently than its reaction with proteins, is biochemically dramatic. Nitrous acid is the most active point mutagen known; it is followed by hydroxyl- and methoxylamine, because these render cytidine ambiguous, making it act with high frequency like uridine. The simple alkylating agents (alkyl sulfates) have low mutagenic activity, but the more complex ones and particularly the nitrosoalkyl compunds show, under biological conditions, considerable mutagenesis. With RNA *in vitro*, none of these appears to be of comparable mutagenicity to nitrous acid, with the particular exception of the alkyl nitrosoguanidines, when acting on TMV-RNA in the virus rod. All mutagenic reagents are also potentially lethal due to side reactions and the fact that many mutations are *per se* lethal. However, all reagents that attack nucleic acids, whether mutagenic or not, are potentially lethal and can be used for virus inactivation.

4. CONCLUSIONS

Virus modification reactions may be applied for a variety of reasons. Presently, the most frequent use of these procedures is to determine the location of structural components of a virion in regard to the virion surface or to one another. Tables 2 and 3 summarize much of the data now available for these purposes, and experimental details can be found in the cited papers. Such studies have contributed greatly to our understanding of viral architecture and fine structure.

When specific groups in viral proteins are to be modified, reagents of high selectivity are required. Such reagents and their limitations are listed in Table 4. Specific details of methodology can be found in Means and Feeney (1971). These methods are of particular usefulness when specific biological functions, such as enzymatic actions of viral components, are under study.

It should be reinterated that most reactions that cause rapid loss of viral infectivity involve viral nucleic acids. In terms of classes of reagents, those of greatest affinity for nucleic acids are the alkylating agents, the nucleophilic compounds, and nitrous acid and formaldehyde.

TABLE 4

Preferred Reagents for Modification of Specific Groups in Virus Proteins[a]

Groups (amino acid)	
Amino (lys)	Imido esters (42–44), *N*-succinimidylpropionate (11), *O*-methylisourea (68)[a]
Carboxyl (asp,glu)	Diazoacetates (69)[a] (also cySH), carbodiimides (55)
Disulfide (cys)	Sodium borohydride (20), thiols (56, 57)
Guanidyl (arg)	1,2-Cyclohexanedione (70)[a], diacetyl trimer (71)[a]
Imidazole (his)	Photo-oxidation (also cySH, trp), ethoxyformic anydride (15) (also amino)
Indole (trp)	*N*-Bromosuccinimide (22) (also cySH, tyr), 2-hydroxy-5-nitrobenzyl bromide (72)[a] (also cySH)
Phenolic (tyr)	Tetranitromethane (73) (also cySH), iodine (also his, cySH)
Sulfhydryl (cySH)	*N*-Ethylmaleimide (25), 5,5′-dithiobis (2-nitrobenzoic acid) (74)[a], *o*-iodosobenzoate (53), *p*-mercuribenzoate (52), methyl mercuric salts (51), mercuric chloride
Thioether (met)	Cyanogen bromide (CNBr)

[a] Reagents that seem not to have been used with virions.

ACKNOWLEDGMENTS

The author is indebted to B. Singer for helpful criticism and suggestions, as well as for drawing his attention to important references.

5. REFERENCES

Andronikov, M. L., Velikodvorskaya, G. A., Tchruni, F., and Tikchonenko, T. J., 1974, The biological effects of chemical modification of intraphage DNA by *O*-methyl-hydroxylamine, *J. Mol. Biol.* **8**:3.

Bancroft, J. B., and Smith, D. G., 1975, The effect of dimethyladipimidate on the stability of cowpea chlorotic mottle and brome mosaic viruses, *J. Gen. Virol.* **27**:257.

Beneke, T. W., Habermehl, K.-O., Diefenthal, W., and Buchholz, M., 1977, Iodination of poliovirus capsid proteins, *J. Gen. Virol.* **34**:387.

Bickle, T. A., Hershey, J. W. B., and Traut, R. R., 1972, Spatial arrangement of ribosomal proteins: Reaction of the *Escherichia coli* 30 S subunit with bis-imidoesters, *Proc. Natl. Acad. Sci. USA* **69**:1327.

Blass, J., Bizzini, B., and Raynaud, M., 1965, Mecanisme de la detoxification par le formal, *C. R. Acad. Sci.* **261**:1448.

Boeye, A., 1959, Induction of a mutation in poliovirus by nitrous acid, *Virology* **9**:691.

Bolton, A., E., and Hunter, W. M,. 1973, The labelling of proteins to high specific radioactivities by conjugation to a ^{125}I-containing acylating agent, *Biochem. J.* **133**:529.

Borgert, K., Koschel, K., Tauber, H., and Wecker, E., 1971, Effect of inactivation by hydroxylamine on early functions of poliovirus, *J. Virol.* **8**:1.

Bracha, M., Sagher, D., Brown, A., and Schlesinger, M. J., 1977*a*, The protease inhibitor *p*-nitrophenyl-*p'*-guanidinobenzoate inactivates Sindbis and other enveloped viruses, *Virology* **77**:45.

Bracha, M., Sagher, D., and Schlesinger, M. J., 1977*b*, Reaction of the protease inhibitor *p*-nitrophenyl-*p'*-guanidinobenzoate with Sindbis virus, *Virology* **83**:246.

Brakier, L., and Verly, W. G., 1970, The lethal action of ethyl methanesulfonate, mitrogen mustard and myleran on the T7 coliphage, *Biochim. Biophys. Acta* **213**:296.

Brammer, K. W., 1963, Chemical modification of viral ribonucleic acid. II. Bromination and iodination, *Biochim. Biophys. Acta* **72**:217.

Brewer, G. J., and Singer, S. J., 1974, On the disposition of the proteins of the membrane-containing bacteriophage PM2, *Biochemistry* **13**:3580.

Brown, A., Consigli, R. A., Zabielski, J., and Weil, R., 1974, Effect of β-propiolactone inactivation of polyoma virus on viral functions, *J. Virol.* **14**:840.

Brown, D. M., McNaught, A. D., and Schell, P., 1966, The chemical basis of hydrazine mutagenesis, *Biochem. Biophys. Res. Commun.* **24**:967.

Brown, F., Smale, C. J., and Horzinck, M. C., 1974, Lipid protein organization in vesicular stomatitis and Sindbis viruses, *J. Gen. Virol.* **22**:455.

Brusick, D. J., 1977, The genetic properties of beta-propiolactone, *Mutat. Res.* **39**:241.

Budowsky, E. I., and Pashneva, N. N., 1971, Mechanism of the mutagenic action of hydroxylamine. V. Chemical reactivation of hydroxylamine-inactivated MS-2 phage, *Biochim. Biophys. Acta* **246**:329.

Budowsky, E. I., Krivisky, A. S., Klebanova, L. M., Metlitskaya, A. Z., Turchinsky, M. F., and Savin, F. A., 1974, The action of mutagens on MS2 phage and on its infective RNA. V. Kinetics of the chemical and functional changes of the genome under hydroxylamine treatment, *Mutat. Res.* **24**:245.

Butler, P. J. G., Harris, J. I., Hartley, B. S., and Leberman, R., 1969, The use of maleic anhydride for the reversible blocking of amino groups in polypeptide chains, *Biochem. J.* **112**:679.

Carthew, P., and Martin, S. J., 1974, The iodination of bovine enterovirus particles, *J. Gen. Virol.* **24**:525.

Cartwright, B., Smale, C. J., and Brown, F., 1969, Surface structure of vesicular stomatitis virus, *J. Gen. Virol.* **5**:1.

Carver, D. H., and Seto, D. S. Y., 1968, Viral inactivation by disulfide bond reducing agents, *J. Virol.* **2**:1482.

Cavanagh, D., Sangar, D. V., Rowlands, D. J., and Brown, F., 1977, Immunogenic and cell attachment sites of FMDV: Further evidence for their location in a single capsid polypeptide, *J. Gen. Virol.* **35**:149.

Champness, J. N., Bloomer, A. C., Bricogne, G., Butler, P. J. G., and Klug, A., 1976, The structure of the protein disk of tobacco mosaic virus to 5 Å resolution, *Nature (London)* **259**:20.

Chase, T., Jr., and Shaw, E., 1969, Comparison of the esterase activities of trypsin, plasmin, and thrombin on guanidinobenzoate esters. Titration of the enzymes, *Biochemistry* **8**:2212.

Corbett, T. H., Heidelberger, C., and Dover, W. F., 1970, Determination of the mutagenic activity to bacteriophage T4 of carcinogenic and non-carcinogenic compounds, *Mol. Pharmacol.* **6**:667.

David, G. S., and Reisfeld, R. A., 1974, Protein iodination with solid state lactoperoxidase, *Biochemistry* **13**:1014.

Davies, G. E., and Stark, G. R., 1970, Use of dimethyl suberimidate, a cross-linking reagent, in studying the subunit structure of oligomeric proteins, *Proc. Natl. Acad. Sci. USA* **66**:651.

Dipple, A., and Shooter, K. V., 1974, Chemical modification and biological inactivation of bacteriophage R17 by 7-bromomethylbenz[a]anthracenes, *Biochim. Biophys. Acta* **374**:392.

Dorne, B., and Hirth, L., 1971, The reaction of mercuric ions with encapsidated and isolated RNA of tomato bushy stunt virus, *Biochimie* **53**:469.

Dubovi, J. E., and Wagner, R. R., 1977, Spatial relationships of the proteins of vesicular stomatitis virus: Induction of reversible oligomers by cleavable protein cross-linkers and oxidation, *J. Virol.* **22**:500.

Durham, A. C. H., and Butler, P. J. G., 1975, A prediction of the structure of tobacco mosaic virus protein, *Eur. J. Biochem.* **53**:397.

Eger, R., and Rifkin, D. B., 1977, The preparation and use of pyridoxal [^{32}P] phosphate as a labeling reagent for proteins on the outer surface of membranes, *Biochim. Biophys. Acta* **470**:70.

Ehrenfeld, E., 1974, Interaction of diethylpyrocarbonate with poliovirus double-stranded RNA, *Biochem. Biophys. Res. Commun.* **56**:214.

Esipova, V. V., Lisovskaya, K. V., and Krivisky, A. S., 1978, Photosensitizing effect of 8-methoxypsoralen on bacteriophage s_d, *Genetika* **14**:975.

Everitt, E., Lutter, L., and Philipson, L., 1975, Structural proteins of adenoviruses. XII. Location and neighbor relationship among proteins of adenovirion type 2 as revealed by enzymatic iodination, immunoprecipitation and chemical cross-linking, *Virology* **67**:197.

Fairhead, S. M., Steel, J. S., Wreford, J., and Walker, I. O., 1969, Studies on the properties of tryptophan and tyrosine side chains in tobacco mosaic virus and tobacco mosaic virus protein, *Biochim. Biophys. Acta* **194**:584.

Feldman, M. Y., 1973, Reactions of nucleic acids and nucleoprotein with formaldehyde, *Prog. Nucleic Acid Res. Mol. Biol.* **13**:1.

Fraenkel-Conrat, H., 1954, Reaction of nucleic acid with formaldehyde, *Biochim. Biophys. Acta* **15**:308.

Fraenkel-Conrat, H., 1955, The reaction of tobacco mosaic virus with iodine, *J. Biol. Chem.* **217**:373.

Fraenkel-Conrat, H., 1958, The masked —SH group in tobacco mosaic virus protein, in: *Sulfur in Proteins* (R. Benesch *et al.*, eds.), pp. 339–346, Academic Press, New York.

Fraenkel-Conrat, H., and Colloms, M., 1967, Reactivity of tobacco mosaic virus and its protein toward acetic anhydride, *Biochemistry* **6**:2740.

Fraenkel-Conrat, H., and Olcott, H. S., 1946, Reaction of formaldehyde with proteins. II. Participation of the guanidyl groups and evidence of cross-linking, *J. Am. Chem. Soc.* **68**:34.

Fraenkel-Conrat, H., and Olcott, H. S., 1948*a*, The reaction of formaldehyde with proteins. V. Cross-linking between amino and primary amide or guanidyl groups, *J. Am. Chem. Soc.* **70**:2673.

Fraenkel-Conrat, H., and Olcott, H. S., 1948*b*, Reaction of formaldehyde with proteins. VI. Cross-linking of amino groups with phenol, imidazole, or indole groups, *J. Biol. Chem.* **174**:827.

Fraenkel-Conrat, H., and Sherwood, M., 1967, Reactivity of tyrosine residues of tobacco mosaic virus protein with iodine, *Arch. Biochem. Biophys.* **120**:571.

Fraenkel-Conrat, H., and Singer, B., 1972, The chemical basis for the mutagenicity of hydroxylamine and methoxyamine, *Biochim. Biophys. Acta* **262**:264.

Fraenkel-Conrat, H., Mohammad, A., Ducay, E. D., and Mecham, D. K., 1951, The molecular weight of lysozyme after reduction and alkylation of the disulfide bonds, *J. Am. Chem. Soc.* **73**:625.

Fraenkel-Conrat, H., Staehelin, M., and Crawford, L. V., 1959, Tobacco mosaic virus reconstitution using inactivated nucleic acid, *Proc. Exp. Biol. Med.* **102**:118.

Franklin, R. M., and Wecker, E., 1959, Inactivation of some animal viruses by hydroxylamine and the structure of ribonucleic acid, *Nature (London)* **184**:343.

Gahmberg, C. G., Simons, K., Renkonen, O., and Kääriäinen, L., 1972, Exposure of proteins and lipids in the Semliki Forest virus membrane, *Virology* **50**:259.

Garoff, H., 1974, Cross-linking of the spike glycoproteins in Semlike Forest virus with dimethylsuberimidate, *Virology* **62**:385.

Garoff, H., and Simons, K., 1974, Location of the spike glycoproteins in the Semlike Forest virus membrane, *Proc. Natl. Acad. Sci. USA* **71**:3988.

Gierer, A., and Mundry, K. W., 1958, Production of mutants of tobacco mosaic virus by chemical alteration of its ribonucleic acid *in vitro*, *Nature (London)* **182**:1457.

Graham, J., and Butler, P. J. G., 1978, Location of tyrosine residues in the disk of tobacco-mosaic-virus protein and comparison of the subunit packing with that of the virus, *Eur. J. Biochem.* **83**:523.

Grunberger, D., and Weinstein, I. B., 1979, Biochemical effects of the modification of nucleic acids by certain polycyclic carcinogens, *Prog. Nucleic Acid Res. Mol. Biol.* **23**:106.

Hallick, L. M., Yokota, H. A., Bartholomew, J. C., and Hearst, J. E., 1978, Photochemical addition to the cross-linking reagent 4,5′,8-trimethylpsoralen (trioxsalen) to intracellular and viral Simian virus 40 DNA-histone complexes, *J. Virol.* **27**:127.

Hanson, C. V., 1979, Photochemical inactivation of DNA and RNA viruses by color promazine, *Antimicrob. Agents Chemother.* **15**:461.

Hanson, C. V., Riggs, J. L., and Lennette, E. H., 1978, Photochemical inactivation of DNA and RNA viruses by psoralen derivatives, *J. Gen. Virol.* **40**:345.

Hartman, P. S., Eisenstark, A., and Pauw, P. G., 1979, Inactivation of phage T7 by near-ultraviolet radiation plus hydrogen peroxide: DNA-protein crosslinks prevent DNA injection, *Proc. Natl. Acad. Sci. USA* **76**:3228.

Hayatsu, H., 1977, Co-operative mutagenic actions of bisulfite and nitrogen nucleophiles, *J. Mol. Biol.* **115**:19.

Hayatsu, H., and Shiragami, M., 1979, Reaction of bisulfite with the 5-hydroxymethyl group in pyrimidines and in phage DNAs, *Biochemistry* **18**:632.

Hearst, J. E., and Thiry, L., 1977, The photoinactivation of an RNA animal virus, vesicular stomatitis virus, with the aid of newly synthesized psoralen derivatives, *Nucleic Acids Res.* **4**:1339.

Hinnen, R., Schäfer, R., and Franklin, R. M., 1974, Structure and synthesis of a lipid-containing bacteriophage. Preparation of virus and localization of the structural proteins, *Eur. J. Biochem.* **50**:1.

Høff-Jorgensen, R., and Lund, E., 1972, Studies on the inactivation of viruses by ethylene oxide, *Acta Vet. Scand.* **13**:520.

Hordern, J. S., Leonard, J. D., and Scraba, D. G., 1979, Structure of the Mengo virion. VI. Spatial relationships of the capsid polypeptides as determined by chemical cross-linking analyses, *Virology* **97**:131.

Hung, P. P., 1973, Rescue of chemically inactivated Rous sarcoma virus transforming activity by avian leukosis virus, *Virology* **53**:463.

Hunter, M. J., and Ludwig, M. L., 1962, The reaction of imidoesters with proteins and related small molecules, *J. Am. Chem. Soc.* **84**:3491.

Hunter, W. M., and Greenwood, F. C., 1962, Preparation of iodine-131 labeled human growth hormone of high specific activity, *Nature (London)* **194**:495.

Inoue, H., and Kuriyama, K., Ohno, T., and Okada, Y., 1974, Circular dichrosim and sedimentation studies on the reconstitution of tobacco mosaic virus, *Arch. Biochem. Biophys.* **165**:34.

Kaper, J. M., 1976, Molecular organization and stabilizing forces of simple RNA viruses. V. The role of lysyl residues in the stabilization of cucumber mosaic virus strain S, *Virology* **71**:185.

Kaper, J. M., and Jenifer, F. G., 1968, Studies on the interaction of *p*-mercuribenzoate with turnip yellow mosaic virus. V. Induced ribonuclease sensitivity and degradation of the virion, *Virology* **35**:71.

Karska-Wysocki, B., Thibodeau, L., and Verly, W. G., 1976, Inactivation of the T7 coliphage by monofunctional alkylating agents. Action of phage adsorption and injection of its DNA, *Biochim. Biophys. Acta* **435**:184.

Katz, E., and Margalith, E., 1973, Location of vaccinia virus structure polypeptides on the surface of the virus particle, *J. Gen. Virol.* **18**:381.

Khromov, I. S., Ulanov, B. P., and Tikhonenko, T. I., 1977, The effect of O-β-diethylaminoethylhydroxylamine on T2 bacteriophage, *Vopr. Virusol.* **4**:497.

King, L., and Leberman, R., 1973, Derivatization of carboxyl groups of tobacco mosaic virus with cystamine, *Biochim. Biophys. Acta* **322**:279.

King, L., and Perham, R. N., 1971, Reaction of tobacco mosaic virus with maleic anhydride and some possible applications to X-ray diffraction analysis, *Biochemistry* **10**:981.

Kochetkov, N. K., Budowsky, E. I., and Simukova, N. A., 1962, The reaction of hydroxylamine with ribonucleic acid, *Biochim. Biophys. Acta* **55**:255.

Krivisky, A. S., Belykh, R. A., and Budowsky, E. I., 1973, Mutagenic and inactivating effects of *O*-methylhydroxylamine on bacterophage ϕX174 and its infectious DNA, *Genetika* **9**:105.

Kröger, M., and Singer, B., 1979, Ambiguity and transcriptional errors as a result of methylation of N-1 of purines and N-3 of pyrimidines, *Biochemistry* **18**:3493.

Kudo, I., Miura, A., and Hayatsu, H., 1978, Inactivation of bacteriophage λ during the aerobic oxidation of bisulfite, *Environ. Res.* **16**:205.

Lawley, P. D., Lethbridge, J. H., Edwards, P. A., and Shooter, K. V., 1969, Inactivation of bacteriophage T7 by mono- and difunctional sulphur mustards in relation to cross-linking and depurination of bacteriophage DNA, *J. Mol. Biol.* **39**:181.

Lomant, A. J., and Fairbanks, G., 1976, Chemical probes of extended biological structures: Synthesis and properties of the cleavable protein cross-linking reagent [^{35}S]dithiobis-succinimidyl propionate, *J. Mol. Biol.* **104**:243.

Lonberg-Holm, K., and Butterworth, B. E., 1976, Investigation of the structure of polio- and human rhinovirions through the use of selective chemical reactivity, *Virology* **71**:207.

Lotlikar, P. D., Fukuda, S., and Yamamoto, N., 1972, Effects of the carcinogen 2-acetylaminofluorene and its derivatives on bacteria and bacteriophages, *Mol. Pharmacol.* **8**:645.

Loveless, A., 1959, The influence of radiomimetic substances on deoxyribonucleic acid synthesis and function studied in *E. coli* phage systems. III. Mutation of T2 bacteriphage as a consequence of alkylation *in vitro;* the uniqueness of ethylation, *Proc. R. Soc. London Serv. B* **150**:497.

Loveless, A., and Stock, J. C., 1959*a*, The influence of radiomimetic substances on deoxyribonucleic acid synthesis and function studied in *E. coli* phage systems. I. The nature of the inactivation of T2 phage *in vitro* by certain alkylating agents, *Proc. R. Soc. London Ser.B* **150**:423.

Loveless, A., and Stock, J. C., 1959*b*, The influence of radiomimetic substances on deoxyribonucleic acid synthesis and function studied in *E. coli* phage systems. II. Properties of coliphage T2 treated with di(2-chloroethyl) methylamine, *Proc. R. Soc. London Ser. B* **150**:486.

Loveless, A., and Stock, J. C., 1959*c*, The influence of radiomimetic substances on deoxyribonucleic acid synthesis and function studied in *E. coli* phage systems. V. A comparison of the inactivation of phages T2, T7 and two strains of T4 by alkylating agents, *Proc. R. Soc. London Ser. B* **151**:148.

Lubeck, M. D., and Johnson, F. B., 1977, Radioiodination of adenovirus-associated virus external structural proteins, *Virology* **83**:453.

Ludlum, D. B., and Wilhelm, R. C., 1968, Ribonucleic acid polymerase reactions with methylated polycytidylic acid templates, *J. Biol. Chem.* **243**:2750.

Lund, G. A., Ziola, B. R., Salmi, A., and Scraba, D. G., 1977, Structure of the Mengo virion. V. Distribution of the capsid polypeptides with respect to the surface of the virus particle, *Virology* **78**:35.

Lutter, L. C., Ortanderl, F., and Fasold, H., 1974, The use of new series of cleavable protein-crosslinkers on the *Escherichia coli* ribosome, *FEBS Lett.* **48**:288.

Luukkonen, A., Gahmberg, C. G., and Renkonen, O., 1977, Surface labeling of Semliki Forest virus glycoproteins using galactose oxidase, *Virology* **76**:55.

Lytle, C. D., and Ginoza, W., 1970, Inactivation of bacteriophage ϕX174 by sublethal nitrous acid-produced lesions, *Biochem. Biophys. Res. Commun.* **39**:809.

Maes, R., and Mesquita, J., 1970, High mutation rate of foot-and-mouth disease virus treated with hydroxylamine, *Arch. Gesamte Virusforsch.* **29**:77.

Mahoney, W. C., and Hermodson, M. A., 1979, High yield cleavage of tryptophanyl peptide bonds by *O*-iodosobenzoic acid, *Biochemistry* **18**:3810.

Marchalonis, J. J., 1969, An enzymic method for the trace iodination of immunoglobulins and other proteins, *Biochem. J.* **113**:299.

Markwell, M. A. K., and Fox, C. F., 1978, Surface-specific iodination of membrane proteins of viruses and eucaryotic cells using 1,3,4,6-tetrachloro-3a,6a-diphenylglycoluril, *Biochemistry* **17**:4807.

McSharry, J. J., 1977, The effect of chemical and physical treatments on the lactoperoxidase-catalyzed iodination of vesicular stomatitis virus, *Virology* **83**:482.

McSharry, J. J., Compans, R. W., and Choppin, P. W., 1971, Proteins of vesicular stomatitis virus and of phenotypically mixed vesicular stomatitis virus-simian virus 5 virions, *J. Virol.* **8**:722.

Means, G. E., and Feeney, R. E., 1968, Reductive alkylation of amino groups in proteins, *Biochemistry* **7**:2192.

Means, G. E., and Feeney, R. E., 1971, *Chemical Modification of Proteins*, pp. 1–254, Holden-Day, San Francisco.

Means, G. E., and Fraenkel-Conrat, H., 1971, Effects of bromine on the template and messenger specificities of polynucleotides, *Biochim. Biophys. Acta* **247**:441.

Montelaro, R. C., and Rueckert, R. R., 1975, Radiolabeling of proteins and viruses *in vitro* by acetylation with radioactive acetic anhydride, *J. Biol. Chem.* **250**:1413.

Mudd, J. A., and Swanson, R. E., 1978, *In situ* cross-linking of vesicular stomatitis virus proteins with reversible agents, *Virology* **88**:263.

Mundry, K. W., and Gierer, A., 1958, Die Erzeugung von Mutationen des Tabakmosaikvirus durch Chemische Behandlung Seiner Nucleinsaure *in vitro*, *Z. Vererbungsl.* **89**:614.

Nagai, Y., Yoshida, T., Hamaguchi, M., Iinuma, M., Maeno, K., and Matsumoto, T., 1978, Cross-linking of Newcastle disease virus (NDV) proteins, *Arch. Virol.* **58**:15.

Nakashima, K., and Shatkin, A. J., 1978, Photochemical cross-linking of reovirus genome RNA *in situ* and inactivation of viral transcriptase, *J. Biol. Chem.* **253**:8680.

Newlin, G. E., and Bussell, R. H., 1975, Characterization of inactivation of myxoviruses and paramyxoviruses by hydroxylamine, *N*-methylhydroxylamine and *O*-methylhydroxylamine, *Arch. Virol.* **47**:97.

Ohno, T., Yamaura, R., Kuriyama, K., Inoue, H., and Okada, Y., 1972, Structure of *N*-bromosuccinimide-modified tobacco mosaic virus protein and its function in the reconstitution process, *Virology* **50**:76.

Okada, Y., Ohno, T., Kuriyana, K., and Inoue, H., 1972, Participation of the tyrosine residue at position 139 of tobacco mosaic virus protein in the reconstitution reaction, *Virology* **47**:838.

Oxelfelt, P., and Arstrand, K., 1970, Effects of diethyl pyrocarbonate on tobacco mosaic virus and its RNA, *Biochim. Biophys. Acta* **217**:544.

Perham, R. N., 1973, The reactivity of functional groups as a probe for investigating the topography of tobacco mosaic virus, *Biochem. J.* **131**:119.

Perham, R. N., and Richards, F. M., 1968, Reactivity and structural role of protein amino groups in tobacco mosaic virus, *J. Mol. Biol.* **33**:795.

Perham, R. N., and Thomas, J. O., 1971, Reaction of tobacco mosaic virus with a thiol-containing imidoester and a possible application to X-ray diffraction analysis, *J. Mol. Biol.* **62**:415.

Philipson, L., 1964, Degradation of polio virions by mercurials, *Biochem. Biophys. Res. Commun.* **17**:352.

Phillips, D. R., and Morrison, M., 1970, The arrangement of proteins in the human erythrocyte membrane, *Biochem. Biophys. Res. Commun.* **40**:284.

Pierpoint, W. S., 1973, PVX-Q: An infective product of potato virus X and a lead o-quinone, *J. Gen. Virol.* **19**:189.

Pierpoint, W. S., 1974, Chemical modification of the lysine-amino groups of potato virus X, *J. Gen. Virol.* **25**:303.

Pierpoint, W. S., and Carpenter, J. M., 1978, An infective pyridoxyl-derivative of potato virus X: PVX-PLP, *J. Gen. Virol.* **38**:505.

Piette, J., Calberg-Bacq, C. M., and Van de Vorst, A., 1978, Photodynamic effect of proflavine on ϕX174 bacteriophage, its DNA replicative form and its isolated single-stranded DNA: Inactivation, mutagenesis and repair, *Mol. Gen. Genet.* **167**:95.

Pinter, A., and Fleissner, E., 1979, Structural studies of retroviruses: Characterization of oligomeric complexes of murine and feline leukemia virus envelope and core components formed upon cross-linking, *J. Virol.* **30**:157.

Ray, U., Bartenstein, L., and Drake, J. W., 1972, Inactivation of bacteriophage T4 by ethyl methanesulfonate: Influence of host and viral genotypes, *J. Virol.* **9**:440.

Rice, R. H., and Means, G. E., 1971, Radioactive labeling of proteins *in vitro*, *J. Biol. Chem.* **246**:831.

Richardson, C. D., and Vance, D. E., 1978, Chemical cross-linking of proteins of Semlike Forest virus: Virus particles and plasma membranes from BHK 21 cells treated with colchicine or dibucaine, *J. Virol.* **28**:193.

Robinson, D. J., 1973, Inactivation and mutagenesis of tobacco rattle virus by nitrous acid, *J. Gen. Virol.* **18**:215.

Rothman, J. E., Tsai, D. K., Dawidowicz, E. A., and Lenard, J., 1976, Transbilayer phospholipid asymmetry and its maintenance in the membrane of influenza virus, *Biochemistry* **15**:2361.

Rushizky, G. W., and Mozejko, J. H., 1973, Preparation of large oligonucleotides by RNase T1 partial hydrolysis of MS2 virus treated with succinic anhydride, *Prep. Biochem.* **3**:425.

Salk, J. E., 1955, Considerations in the preparation and use of poliomyelitis virus vaccines, *J. Am. Med. Assoc.* **158**:1239.

Salk, J. E., and Gori, J. B., 1960, A review of theoretical, experimental, and practical considerations in the use of formaldehyde for the inactivaton of poliovirus, *Ann. N.Y. Acad. Sci.* **83**:609.

Sarov, I., and Joklik, W. K.,1972, Studies on the nature and location of the capsid polypeptides of vaccinia virions, *Virology* **50**:579.

Schäfer, R., Huber, U., Franklin, R. M., and Seelig, J., 1975, Structure and synthesis of a lipid-containing bacteriophage. Chemical modifications of bacteriophage PM2 and the resulting alterations in acyl-chain motion in the PM2 membrane, *Eur. J. Biochem.* **58**:291.

Scheele, R. B., and Lauffer, M. A., 1969, Restricted reactivity of the ϵ-amino groups of tobacco mosaic virus protein toward trinitrobenzenesulfonic acid, *Biochemistry* **8**:3597.

Schuster, H., and Schramm, G., 1958, Bestimmung der Biologisch Wirksamen Einheit in der Ribosenucleinsaure des Tabakmosaikvirus auf Chemischem Wege, *Z. Naturforsch.* **13b**:698.

Schuster, H., and Wilhelm, R. C., 1963, Reaction difference between tobacco mosaic virus and its free ribonucleic acid with nitrous acid, *Biochim. Biophys. Acta* **68**:554.

Sefton, B. M., Wickus, G. G., and Burge, B. W., 1973, Enzymatic iodination of Sindbis virus proteins, *J. Virol.* **11**:730.

Sehgal, O. P., 1973, Inactivation of Southern bean mosaic virus and its ribonucleic acid by nitrous acid and ultraviolet light, *J. Gen. Virol.* **18**:1.

Sehgal, O. P., and Hsu, C. H., 1977, Identity and location of a minor protein component in virions of Southern bean mosaic virus, *Virology* **77**:1.

Sehgal, O. P., and Krause, G. F., 1968, Efficiency of nitrous acid as an inactivating and mutagenic agent of intact tobacco mosaic virus and its isolated nucleic acid, *J. Virol.* **2**:966.

Shapiro, R., 1964, Isolation of 2-nitropurine from the reaction of guanosine with nitrous acid, *J. Am. Chem. Soc.* **86**:2948.

Shapiro, R., Dubelman, S., Feinberg, A., Crain, P. F., and McCloskey, J. A., 1977, Isolation and identification of crosslinked nucleosides from nitrous acid treated DNA, *J. Am. Chem. Soc.* **99**:302.

Shen, C,-K. J., Hsieh, T-S., Wang, J. C., and Hearst, J. E., 1977, Photochemical crosslinking of DNA-RNA helices by psoralen derivatives, *J. Mol. Biol.* **116**:661.

Shen, C.-K. J., Ikoku, A., and Hearst, J. E., 1979, A specific DNA orientation in the filamentous bacteriophage fd as probed by psoralen crosslinking and electron microscopy, *J. Mol. Biol.* **127**:163.

Shooter, K. V., 1975, Assays for phosphotriester formation in the reaction of bacteriophage R17 with a group of alkylating agents, *Chem. Biol. Interact.* **11**:575.

Shooter, K. V., and Howse, R., 1975, The inactivation of bacteriophage R17 by ethylating agents: The lethal lesions, *Chem. Biol. Interact.* **11**:563.

Shooter, K. V., and Wiessler, M., 1976, The interaction of acetoxy-dimethylnitrosamine, a proximate metabolite of the carcinogenic amine, and bacteriophages R17 and T7, *Chem. Biol. Interact.* **14**:1.

Shooter, K. V., Edwards, P. A., and Lawley, P. D., 1971, The action of mono and difunctional sulphur mustards on the ribonucleic acid-containing bacteriophage $\mu 2$, *Biochem. J.* **125**:829.

Shooter, K. V., Howse, R., and Merrifield, R. K., 1974*a*, The reaction of alkylating agents with bacteriophage R17, Biological effects of phosphotriester formation, *Biochem. J.* **137**:313.

Shooter, K. V., Howse, R., Shah, S. A., and Lawley, P. D., 1974*b*, The molecular basis for biological inactivation of nucleic acids. The action of methylating agents on the ribonucleic acid-containing bacteriophage R17, *Biochem. J.* **137**:303.

Siezen, R. J., 1979, Crosslinking of β-crystallin with bisimidoesters. Evidence for polyamidine formation at pH 8 from an increase in positive charges on the polypeptide chains, *FEBS Lett.* **100**:75.

Singer, B., 1975, The chemical effects of nucleic acid alkylation and their relationship to mutagenesis and carcinogenesis, *Prog. Nucleic Acid Res. Mol. Biol.* **15**:219, 330.

Singer, B., 1976, All oxygens in nucleic acids react with carcinogenic ethylating agents, *Nature* (*London*) **264**:333.

Singer, B., 1977, Nucleic acid alkylation, mutation and carcinogenesis: Is there a relationship? *Trends Biochem. Sci.* **2**:180.

Singer, B., and Fraenkel-Conrat, H., 1969*a*, Chemical modification of viral ribonucleic acid. VIII. The chemical and biological effects of methylating agents and nitrosoguanidien on tobacco mosaic virus, *Biochemistry* **8**:3266.

Singer, B., and Fraenkel-Conrat, H., 1969*b*, Mutagenicity of alkyl and nitrosoalkyl compounds acting on tobacco mosaic virus and its RNA, *Virology* **39**:395.

Singer, B., and Fraenkel-Conrat, H., 1970, Messenger and template activities of chemically modified polynucleotides, *Biochemistry* **9**:3694.

Singer, B., Fraenkel-Conrat, H., Greenberg, J., and Michelson, A. M., 1968, Reaction of nitrosoguanidine (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine) with tobacco mosaic virus and its RNA, *Science* **160**:1235.

Singer, B., Bodell, W. J., Cleaver, J. E., Thomas, G. H., Rajewsky, M. F., and Thon, W., 1978, Oxygens in DNA are main targets for ethylnitrosourea in normal and xeroderma pigmentosum fibroblasts and fetal rat brain cells, *Nature* (*London*) **276**:85.

Singer, B., Pergolizzi, R. G., and Grunberger, D., 1979, Synthesis and coding

properties of dinucleoside diphosphates containing alkyl pyrimidines which are formed by the action of carcinogens on nucleic acids, *Nucleic Acids. Res.* **6**:1709.

Singer, B., Pulkrabek, P., Weinstein, I. B., and Grunberger, D., 1980, Infectivity and reconstitution of TMV RNA with *N*-acetoxy-2-acetylaminofluorene or benzo(*a*)pyrene 7,8-dihydrodiol-9,10-oxide, *Nucleic Acid Res.* **8**:2067.

Singh, M. K., and Terzaghi, E., 1974, Selective blockage of bacteriophage T4 assembly by chemical modification, *Nature* (*London*) **252**:321.

Sklyadneva, V. B., Kisseleva, N. P., Budowsky, E. I., and Tikchonenko, T. I., 1970, The reaction of *O*-methyldroxylamine with DNA in solution and inside the phage particles, *Mol. Biol.* **4**:109.

Sklyadneva, V. B., Chekanovskaya, L. A., Nikolaeva, I. A., and Tikchonenko, T. I., 1978, Study of interaction between sodium bisulfite and bacteriophage S_D DNA, *Biokhimiya* **43**:1563.

Smith, B. J., 1976, Inactivation and mutation of coliphage T4 by aliphatic nitrosamides and methanesulphonates: *In vitro* recovery of infectivity of T4 inactivated by isopropyl methanesulphonate, *Muta. Res.* **36**:265.

Staehelin, M., 1959, Inactivation of virus nucleic acids with glyoxal derivatives, *Biochim. Biophys. Acta* **31**:448.

Staehelin, M., 1960, Chemical modifications of virus infectivity: Reactions of tobacco mosaic virus and its nucleic acid, *Experientia* **16**:473.

Stanley, P., and Haslam, E. A., 1971, The polypeptides of influenze virus. V. Localization of polypeptides in the virion by iodination techniques, *Virology* **46**:764.

Sturman, L. S., 1977, Characterization of a coronavirus. I. Structural proteins: Effects of preparative conditions on the migration of proteins in polyacrylamide gels, *Virology* **77**:637.

Thomas, M. J. A., and Hannoun, C., 1957, Analyse de L'action inhibitrice du glyoxal sur la multiplication du virus de la grippe, *C. R. Seances Acad. Sci.* **244**:2258.

Thomas, M. J. A., Barru, E., and Cocioba, I. D., 1957, Action due glyoxal sur les bacteriophages et sur les acides nucleiques, *C. R. Seances Acad. Sci.* **245**:1182.

Tikchonenko, T. I., Budowsky, E. I., Sklyadneva, V. B., and Khromov, I. S., 1971, The secondary structure of bacteriophage DNA *in situ*. III. Reaction of S_d phage with *O*-methylhydroxylamine, *J. Mol. Biol.* **55**:535.

Tikchonenko, T. I., Kisseleva, N. P., Zintshcnko, A. I., Ulanov, B. P., and Budowsky, E. I., 1973, Peculiarities of the secondary structure of bacteriophage DNA *in situ*, *J. Mol. Biol.* **73**:109.

Traut, R. R., Bollen, A., Sun, T.-T., Hershey, J. W. B., Sundberg, J., and Pierce, L. R., 1973, Methyl 4-mercaptobutyrimidate as a cleavable cross-linking reagent and its application to the Escherichia coli 30S ribosome, *Biochemistry* **12**:3266.

Turchinsky, M. F., Kusova, K. S., and Budowsky, E. I., 1974, Conversion of noncovalent interactions in nucleoproteins into covalent bonds: Bisulfite-induced formation of polynucleotide-protein crosslinks in MS2 bacteriophage virions, *FEBS Lett.* **38**:304.

Van Etten, J., Lane, L., Gonzalez, C., Partridge, J., and Vidaver, A., 1976, Comparative properties of bacteriophage $\phi 6$ and $\phi 6$ nucleocapsid, *J. Virol.* **18**:652.

Venitt, S., and Shooter, K. V., 1972, The role of DNA cross-linking in the inactivation of bacteriophage T7 by carcinogenic bromomethylbenz(a)-anthracenes, *Biochim. Biophys. Acta* **277**:479.

von Heicken, K., and Spicher, G., 1959, Uber die Kinetik der Inaktivierung und

Realtivierung von Phagen. III. Mitt: Trefferthroretische Analyse des Inaktivierungsvorganges von Phagen durch Formaldehyd, *Z. Naturforsch.* **14b**:318.

von Vielmetter, W., and Schuster, H., 1960, Die basenspezifitat bei der induktion von mutationen durch salpetrige saure im phagen T2, *Z. Naturforsch.* **15b**:304.

von Vielmetter, W., and Wieder, C. M., 1959, Mutagene und inaktivierende wirkung salpetriger saure auf freie partikel des phagen T2, *Z. Naturforsch.* **14b**:312.

Vorob'eva, A. M., Kul'skii, L. A., and Smirnova, R. S., 1978, Inactivation of coxsackie B viruses with sodium hypochlorite, *Hyg. Sanit. USSR* **8**:96.

Walter, G., and Mudd, J. A., 1973, Iodination of vesicular stomatitis virus with lactoperoxidase, *Virology* **52**:574.

Wetz, K., and Habermehl, K.-O., 1979, Topographical studies on poliovirus capsid proteins by chemical modification and cross-linking with bifunctional reagents, *J. Gen. Virol.* **44**:525.

Wiley, D. C., Skehel, J. J., and Waterfield, M., 1977, Evidence from studies with cross-linking reagent that the haemagglutin of influenza virus is a trimer, *Virology* **79**:446.

Witte, O. N., Weissman, I. L., and Kaplan, H. S., 1973, Structural characteristics of some murine RNA tumor viruses studied by lactoperoxidase iodination, *Proc. Natl. Acad. Sci. USA* **70**:36.

Yamamoto, K., and Kondo, S., 1978, Mechanism of potent mutagenic action of *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine on intracellular phage lambda, *J. Mol. Biol.* **118**:413.

Young, D. C., Johnson, J. D., and Sharp, D. G., 1977, The complex reaction kinetics of ECHO-1 virus with chlorine in water (39965), *Proc. Soc. Exp. Biol. Med.* **156**:496.

CHAPTER 6

Photobiology of RNA Viruses

Terence M. Murphy

Department of Botany
University of California
Davis, California 95616

and

Milton P. Gordon

Department of Biochemistry
University of Washington
Seattle, Washington 98195

1. INTRODUCTION*

1.1. Scope

Studies on the photochemistry and photobiology of viruses have paralleled studies on their structure, replication, and genetic organization. Nonionizing radiation, because of its relative specificity of action, has served as a dissecting tool in many types of investigations.

* Abbreviations: RNA, ribonucleic acid; PVX, potato virus X; RSV, rous sarcoma virus; TMV, tobacco mosaic virus; TNV, tobacco necrosis virus; TRV, tobacco rattle virus; VSV, vesicular stomatitis virus; UV, ultraviolet radiation; DI, defective interfering.

RNA viruses provide interesting material for photobiological studies. On the one hand, they may be used as material for general investigations into the photochemistry of nucleic acids and nucleic acid–protein complexes; they form models for studying effects of radiation on forms of cellular RNA. On the other hand, RNA viruses are pathogenic agents of recognized importance. It is hoped that radiation studies may contribute to the understanding and control of these agents.

Photochemical techniques have been applied to many types of virus, but their use has varied according to the chemical and physical peculiarities of viruses and the characteristics of their hosts.

Plant RNA viruses, which have been available in large quantities for many years, have been chemically analyzed to determine UV-induced photoproducts in RNA. They have also been used as model systems to study the effect of nucleic acid–protein complexing on the photochemistry of each component. They have been used to study RNA photorepair, a peculiarity of plants. And in a few investigations, they have been irradiated with a view toward understanding the mechanism of their infectivity and replication.

Bacterial RNA viruses, too, have been analyzed chemically following irradiation. With bacterial viruses, the relative simplicity of the virus–host and virus–transcriptase systems have made these systems useful in studying the effects of photoproducts on RNA function.

Animal RNA viruses also have been used for photochemical studies in which the gross and detailed events accompanying loss of infectivity and antigenicity are defined. More recently, the information available concerning the genetics and biochemistry of animal virus replication has greatly expanded. Radiation studies have been used to develop, and have built on, such information. For instance, ultraviolet radiation has been used to distinguish between genetic components needed for infectivity and those needed for shutoff of host cell activities in poliovirus and vesicular stomatitis virus. With VSV and C viruses, radiation has played a role in studies of genetic organization. The inactivation and repair of double-stranded RNA has been studied by UV irradiation of the replicating form of encephalomyocarditis virus.

1.2. Photochemical Background

Both nucleic acid and protein components of RNA viruses absorb light in the ultraviolet region of the spectrum and react when irradiated by UV. In this section we describe some observations with simple

model compounds which suggest possible photochemical reactions that might occur in viruses.

1.2.1. Ribonucleic Acids

When the free bases of RNA or their nucleosides are irradiated at 254 nm, the photoproducts that can be identified include (Fig. 1) hydrates of cytosine and uracil (5,6-dihydro-6-hydroxyl-cytosine and -uracil), cyclobutadipyrimidines, and pyrimidine–pyrimidine adducts and their secondary products (6-4′-(pyrimidin-2′-one)uracil). There are two possible isomers of each hydrate, but in practice they generally are not resolved. There are four possible isomers of cyclobutadipyrimidines (*cis-syn*, *cis-anti*, *trans-syn*, *trans-anti*), which can easily be resolved. The distribution of products depends strongly on solution conditions. Other pyrimidine photoproducts formed under special circumstances include oxetanes, formed by addition of ketones across the 5,6 double bond, and cyclobutane products, formed by addition of olefins across the same bond. Purine bases are about 10 times less sensitive to UV than pyrimidine bases under normal conditions (Varghese, 1973).

In polymers, RNA bases undergo many of the same reactions described above. UV irradiation of poly(rU) produces hydrates and

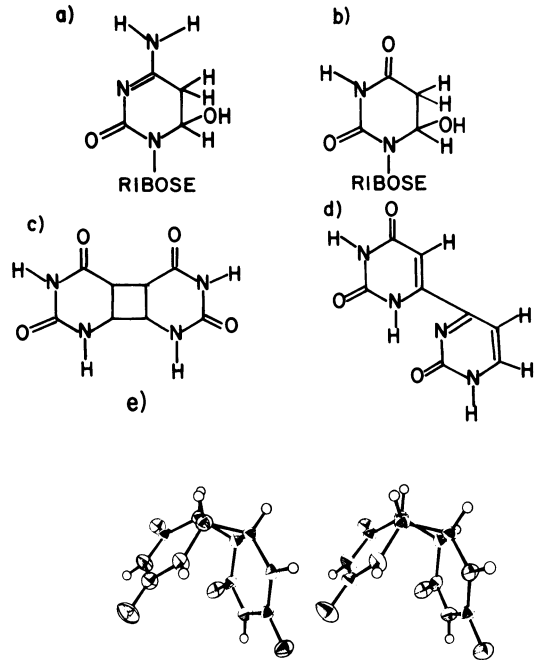


Fig. 1. Photoproducts of UV-irradiated RNA components. a, Cytidine hydrate; b, uridine hydrate; c, (*syn*)-cyclobutadiuracil; d, uracil–uracil adduct; e, (*cis*, *syn*)-cyclobutadiuracil. From Adman *et al.* (1968); stereopair courtesy of E. Adman.

cyclobutane-type dimers (three isomers) (Pearson and Johns, 1966). Irradiation of poly(C) gives products 85% of which are apparently hydrates (Ono *et al.*, 1965). Cytosine–cytosine adducts are also formed in poly(rC): Rhoades and Wang (1971) identified the deamination product of an adduct.

The amounts and types of photoproducts formed in irradiated polynucleotides depend strongly on the characteristics of the irradiated molecule and on the solvent and temperature. In part, this dependence is due to the ability of polyribonucleotides to shift between single-stranded and double-stranded helical forms. The rate of formation both of uridine hydrate and of cyclobutadiuridine is 5 and 10 times less, respectively, in double-stranded poly(rU):poly(rA) than in single-stranded poly(rU) (Pearson and Johns, 1966) and is less in an ordered form of poly(U) than in a disordered form (DeBoer *et al.*, 1967; Lomont and Fresco, 1972). The major photoproduct in double-stranded poly(rC):poly(rI) is cyclobutane-type dimer (Setlow *et al.*, 1965), in contrast to the hydrates formed in poly(rC). Similar effects may influence the photochemistry of viral RNA: for instance, high ionic strength causes an increase of helicity in tobacco mosaic virus RNA (Boedtker, 1960; McMullen *et al.*, 1967).

The detection and accurate quantitation of photoproducts in RNA poses serious problems. Both pyrimidine hydrates and cyclobutadipyrimidines are unstable under conditions that might be used in the analysis of RNA. Hydrates of uracil and uracil derivatives dehydrate; this reaction is speeded by heat, acid, and base, and is fastest for nucleosides and nucleotides (Logan and Whitmore, 1966). Hydrates of cytosine and cytosine derivatives dehydrate, or they deaminate to form corresponding uracil-hydrate compounds; the deamination reaction is accelerated by the presence of salts (DeBoer *et al.*, 1970). Some isomers of cyclobutadiuracil formed in frozen solution are degraded by conditions of acid hydrolysis (Setlow *et al.*, 1965), as well as by base (Varghese, 1973). Cyclobutadiuracil and uracil hydrate that are formed in ^{32}P-labeled RNA can be degraded by ring opening in alkali (Schuster, 1964). Cyclobutadicytosine deaminates to cyclobutadiuracil (Varghese, 1972), and cyclobutadicytidine, upon heating at 100°C, forms cytidine (at pH 2–4), cyclobutadiuridine (pH 4–8), or uridine (pH 10–12) (Varghese and Rupert, 1971). It is thus difficult to recover high proportions of pyrimidine hydrates or cyclobutadipyrimidines formed by UV in RNA, especially if heat, acid, base, or long incubations are involved.

In addition, hydrates of cytosine and uracil exchange their 5-hydrogens with the solvent (Wechter and Smith, 1968). Thus hydrates

of pyrimidines labeled with tritium at this position will lose their identifying label, even if they retain their identity.

Two analytical techniques have been developed to overcome these problems. One involves the reductive cleavage of pyrimidine photohydrates by $NaBH_4$. Reduction of cytidine hydrate forms N_1-(α,β-D-ribopyranosyl)-N_3-(γ-hydroxypropyl) urea; reduction of uridine hydrate forms α,β-D-ribopyranosyl urea and 1,3-propanediol (Miller and Cerutti, 1968). Incubation of irradiated RNA with [^{3}H]-$NaBH_4$ reduces uridine hydrate and releases [^{3}H]-1,3-propanediol without any need for labeling or digestion of the RNA. The 1,3-propanediol is easily separated from the RNA by ion exchange and thin-layer chromatography and is taken as a quantitative measure of the uridine hydrate formed by irradiation (Cerutti *et al.*, 1969).

The second technique involves enzymatic digestion of labeled, irradiated polyribonucleotides. Pearson and Johns (1966) hydrolyzed [^{32}P]-poly(rU) with pancreatic ribonuclease and separated the products by paper chromatography. The products included Up, Hp, DpUp, and DpHp, where D and H stand for cyclobutadiuridine and uridine hydrate, respectively. Two isomers of cyclobutadiuridine were found. Other enzymes, bacterial alkaline phosphatase and snake venom and spleen phosphodiesterases, were used to prove the identity of the products. Small *et al.* (1968) streamlined this method and applied it to TMV-RNA. They prepared tritium-labeled RNA and digested it with a mixture of pancreatic ribonuclease, spleen and snake venom phosphodiesterases, alkaline phosphatase, and micrococcal nuclease. Photoproducts in irradiated RNA could be separated by paper chromatography. Although the temperature of incubation is low (30°C), the long incubation time (24 hr) leads to loss of photoproduct, especially hydrate. The authors added unlabeled uridine hydrate to the incubation mixture as a control for recovery.

More detailed information on the photochemistry of RNA can be found in an article by Varghese (1973) and Chapters 3–7 in Volume I of Wang (1976).

1.2.2. Proteins

The amino acids that can be directly photolyzed by ultraviolet radiation (254 nm) include cystine (—S—S—), tryptophan, phenylalanine, and tyrosine. The relative sensitivities of these amino acids, calculated by the product of their molar absorptivity and quantum yield for

photolysis at 254 nm, have been estimated as cys, 35.1, trp, 11.5, phe, 1.8, and tyr, 0.6; the peptide bond of acetyl-alanine has a sensitivity of 0.01; histidine, <0.0072 (McLaren and Shugar, 1964).

The products from photolysis of amino acids are diverse and depend strongly on the conditions of irradiation. The irradiation of cystine yields cysteine and H_2S; tryptophan yields serine, aspartic acid, kynurenine, and formyl-kynurenine, among other products; phenylalanine yields tyrosine and 3,4-dihydroxyphenylalanine; and tyrosine yields 3,4-dihydroxyphenylalanine (McLaren and Shugar, 1964). Flash photolysis experiments with laser light (265 nm) have identified initial events in the photolysis of aromatic amino acids. Under proper conditions, tryptophan, phenylalanine, and tyrosine are all capable of ejecting hydrated electrons and forming radicals. The radicals are unstable, and the hydrated electrons promote further reactions. Singlet and triplet energy from these amino acids also can be transferred directly to disulfides (see Laustriat and Hasselmann, 1975, and Lee and Fugate, 1978, for references).

It is more difficult to determine the photoreactions of amino acid residues in proteins and to estimate their relative significance to protein function. Early studies indicated that the sensitivities of several enzymes to ultraviolet-induced inactivation were directly related to their total contents of sensitive amino acids (cysteine, histidine, phenylalanine, tryptophan, tyrosine) (McLaren and Hidalgo-Salvatierra, 1964). These authors interpreted this observation in terms of independent chromophores, and suggested that a change in any sensitive amino acid inactivates the enzyme. Other workers ascribed a major role in the inactivation of functional proteins to the photolysis of cystine (Setlow, 1955; Augenstein and Riley, 1964). In this case, the relationship between sensitivity and number of sensitive amino acids could represent the absorption of light energy and its transfer to cystine. More recent work on the inactivation of specific enzymes suggests that the initial processes and the sensitive sites will differ in each protein. Photolysis of tryptophan inactivates lysozyme (Grossweiner and Usui, 1971) and papain (Baugher and Grossweiner, 1975). Photolysis of cystine, through an energy transfer from tyrosine or tryptophan, inactivates trypsin and RNase A (Kaluskar and Grossweiner, 1974; Volkert and Grossweiner, 1973; Ramachandran and Ghiron, 1979). See Grossweiner (1976) and Laustriat and Hasselmann (1975) for reviews.

Viral proteins may modify the photochemical reactivity of viral RNA (Gordon, 1976). A model for such an interaction may be found in the photochemical addition of amino acids to pyrimidines in irradiated

solutions (Smith and O'Leary, 1967; Smith and Neun, 1968; see Smith, 1976,*a*,*b*, and Kornhauser, 1976, for recent reviews). A cysteine-uracil adduct, 5-*S*-cysteine-6-hydrouracil, has been isolated from an irradiated solution of the two components (Smith and Alpin, 1966; Jellinek and Johns, 1970). Other models demonstrating interactions include the photosensitized formation of cyclobutadithymine by tyrosine in aqueous solution (Kaneko *et al.*, 1978) and the sensitization of the splitting of cyclobutadipyrimidines by tryptophan (Heléne and Charlier, 1971; Chen *et al.*, 1976).

The photochemistry of model compounds demonstrates reactions to which effects of light on viruses may be related. The actual effects, however, are modified by the detailed structure and biology of individual viruses as well as by the conditions of treatment, and thus are quite diverse.

2. PLANT VIRUSES

2.1. Rigid-Rod Viruses: TMV

2.1.1. General

Rigid-rod viruses, typified by tobacco mosaic virus (TMV), consist of a single type of single-stranded RNA (molecular weight 2×10^6) and a single type of protein subunit (molecular weight 17,500). The protein subunits (2130 per virus) and the RNA are combined and wound into a helix: 130 turns with a pitch of 2.3 nm form a cylinder of length 300 nm and diameter 15–18 nm, with an internal channel 4 nm in diameter. The RNA is buried within the protein cylinder. It forms a helix of diameter 8 nm and pitch equal to that of the whole virus. There are three nucleotides associated with each protein subunit. (See Volume 1 of this series, p. 97, for further details.)

The aggregation properties of the protein are considered to determine the overall shape of TMV and the configuration of TMV-RNA within the virus particle. Protein subunits that have been purified free of RNA can, under appropriate conditions, form helical cylinders similar to whole virus (Lauffer and Stevens, 1968). The conformation of the RNA within the virus protein is unlike that observed with any free RNA in solution. The exact structure of the RNA within the virus, and the nature of associations between adjacent nucleic acid bases and between bases and proteins, are still matters under study (Tikchonenko, 1975: Vol. 5, p. 1, of this series).

The RNA of the virus contains all the information necessary to establish an infection. Purified RNA is infective. Thus it is possible to compare the effects of ultraviolet radiation on the infective properties of free and protein-coated RNA.

2.1.2. Inactivation of RNA Infectivity

2.1.2a. Action Spectrum

The action spectrum for the inactivation of TMV-RNA closely follows the RNA absorption spectrum (Fig. 2). The inactivation follows single-hit kinetics (Kleczkowski, 1960; McLaren and Shugar, 1964; Goddard *et al.*, 1966), so inactivation rates can be compared by the slopes of regression lines on a semi-logarithmic plot of survival vs. radiation dose. Rushizky *et al.* (1960) measured the quantum yield for inactivation at 230, 248, 253, 265, and 280 nm and found an average value around 3.5×10^{-3}. This value was confirmed by Merriam and Gordon (1965).

Since the absorption spectrum of TMV-RNA extends above 290 nm (OD_{290} = 1/10 of peak OD_{258}), intermediate-wavelength UV (290–315 nm) and perhaps long-wavelength UV (315–400 nm) sources can inactivate this viral RNA even though they are not as effective on an incident energy basis as short-wavelength UV (<290 nm). This means that natural solar UV should inactivate. Murphy (1973*a*) has described the inactivation of TMV-RNA by sunlight. The dependence of the rate of inactivation on time of day and season of year that was found is consistent with calculations regarding the amount of inter-

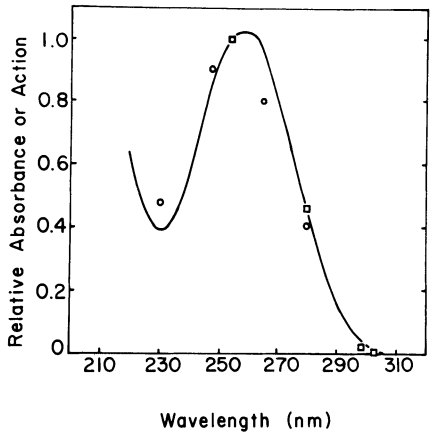


Fig. 2. O, Action spectrum for inactivation of infectivity of TMV-RNA (McLaren and Moring-Claesson, 1961). □, Action spectrum for formation of dipyrimidines in TMV-RNA, calculated as the product of quantum yield for inactivation (Merriam and Gordon, 1965) by the ratio of dipyrimidines per biological hit (Merriam and Gordon, 1967) and by the absorption cross-section for TMV-RNA (McLaren and Takahashi, 1959). ———, Absorption spectrum of TMV (McLaren and Takahashi, 1959). All data normalized to 1.0 at 254 nm.

mediate-wavelength UV sunlight which reaches the Earth's surface (Green *et al.*, 1974) and with an assumed action spectrum similar to the absorption spectrum of TMV-RNA.

2.1.2b. Nature of the Lethal Lesion

It is important but very difficult to identify the lethal photoreactions and photoproducts induced in viral RNA. The difficulty arises because there is often more than one photoproduct formed, and the total number of photoproducts is often considerably greater than the number of biological hits per viral RNA molecule. Experiments to correlate a particular photoproduct with inactivation often involve changes in the wavelength or other conditions of irradiation, but it is possible that the mechanism of inactivation varies with conditions. The small number of photoproducts involved in inactivation at low doses makes quantitation difficult, yet high doses may obscure the photoproducts that are important at low doses. Finally, unmeasured or undetected photoproducts could have important biological effects. These reservations should be considered as part of the following discussion.

The photoproducts that have been detected in UV-irradiated TMV-RNA include cyclobutadipyrimidines (Merriam and Gordon, 1967; Small *et al.*, 1968; Carpenter and Kleczkowski, 1969; Singer, 1971), uridine hydrates (Small *et al.*, 1968; Singer, 1971), and uridine-containing noncyclobutane-type dinucleotides (Small *et al.*, 1968) which may represent pyrimidine–pyrimidine (6-4) adducts (Varghese and Wang, 1968). Under special conditions, other photoproducts have been noted: Evans and McLaren (1968) found the product of HCN addition to uridine, and Tao (1967) found a putative methanol addition product.

The biological and chemical effects of UV can be modified by changing the ionic strength of the solution containing TMV-RNA during irradiation. In general, the cross-section and the quantum yield for inactivation decrease with increasing ionic strength (Lozeron, 1964; Tao, 1967; Merriam and Gordon, 1967; Small *et al.*, 1968; Evans and McLaren, 1969*a*; Singer, 1971). The decrease in biological sensitivity is matched by a decrease in UV-induced conformation changes measured by circular dicroism and thermal melting (Wang and McLaren, 1972), and both decreases may be correlated with an increase in helicity which occurs at high ionic strength (Boedtker, 1960; McMullen *et al.*, 1967). Singer (1971) found that, under her conditions, TMV-RNA irradiated in distilled water formed two uridine hydrates per one uridine-contain-

ing cyclobutadipyrimidine, while the same RNA irradiated in 10^{-3} M Mg^{2+} formed one hydrate per two dipyrimidines. It is quite likely that the change in biological effects of UV induced by changes in ionic strength depends on the change in the number, position, or grouping of hydrates and/or dimers. However, as mentioned above, other photoproducts may be involved.

The inactivation of TMV-RNA also depends on the isotopic composition of the solvent when the ionic strength of the solution is low. The rate of inactivation of TMV-RNA in H_2O is 2.2 times greater than the rate of inactivation in D_2O (99 mole% deuterium oxide) (Tao *et al.*, 1966; Evans and McLaren, 1969*b*). This observation implies that the solvent participates at a rate-limiting step in the UV-dependent reaction sequence leading to inactivation. Solvent clearly participates in the photohydration of uridine, suggesting that uridine hydrates are lethal photoproducts. Small *et al.* (1968) showed that, consistent with the above suggestion, the rate of hydration of uridine in H_2O was 2.2 times that in D_2O.

It is possible that the solvent composition might affect cyclobutadipyrimidine formation, especially the distribution of isomers (Khattak and Wang, 1972). Solvent has been shown to affect the stereochemistry of the photodimerization of coumarin (Morrison *et al.*, 1966) and of the light-induced addition of a cyclopentenone to a cyclohexenone (Challand and DeMayo, 1968). However, the substitution of H_2O by D_2O did not affect the rate of formation of uridine-containing cyclobutadipyrimidines or non-cyclobutane-type dimeric photoproducts in TMV-RNA (Small *et al.*, 1968). There was no indication of a change in isomeric composition of the dimer fractions, either. The rate of inactivation in D_2O was half that in H_2O; thus the number of biological hits per dimer in D_2O was half the number in H_2O. This difference can be explained if, under conditions of low ionic strength, at least half (perhaps a greater fraction) of the dimeric products are not lethal.

At high ionic strength, the rate of inactivation of TMV-RNA is the same in D_2O as in H_2O (Small *et al.*, 1968; Tao, 1967). This suggests that the mechanism of inactivation is different at high ionic strength from that at low ionic strength and that the rate-limiting step in killing at high ionic strength does not involve solvent.

The photoreactions leading to the inactivation of TMV-RNA infectivity can be simplified through the use of intermediate- or long-wavelength UV radiation, together with a triplet sensitizing agent. Various aryl and alkyl ketones, including acetone, acetophenone, and benzophenone, absorb light of wavelengths in the UV-B region and transfer the energy directly to free pyrimidines or to pyrimidines in

nucleic acids through a triplet sensitization mechanism. The result is a relatively efficient production of cyclobutadipyrimidines (Lamola, 1969; Charlier and Heléne, 1972; Varghese, 1972; Charlier *et al.*, 1972; Jennings *et al.*, 1972; Rahn *et al.*, 1974), although benzophenone and acetone also promote the formation of single-strand breaks in DNA, probably through the formation of OH radicals (Charlier *et al.*, 1972; Rahn *et al.*, 1974), and acetophenone causes the production of alkali-labile bonds in DNA by a reaction possibly involving singlet oxygen (Zierenberg *et al.*, 1971; Rahn *et al.*, 1974). Acetone and acetophenone have also been shown to add to uracil and cytosine, forming oxetanes (Varghese, 1972).

Huang and Gordon (1972) inactivated TMV-RNA (and PVX-RNA) by irradiating at 313 nm in the presence of acetone. Cyclobutadipyrimidines were the only detectable photoproducts. No strand breakage or oxetane formation was observed. Dihydrouracil formation was calculated to be negligible. Inactivation of infectivity due to formation of H_2O_2 was shown to be slow and could be controlled by addition of catalase. Thus cyclobutadipyrimidines are probably lethal under these conditions. Murphy (1975*a*,*b*) has shown that acetone strongly sensitizes the inactivation of TMV-RNA infectivity by sunlight. Five percent acetone gave approximately a 160-fold sensitization; 0.5% acetone gave a 40-fold sensitization; 0.05% acetone gave no detectable sensitization. Two other compounds, sulfanilamide and chlortetracycline, also were used to sensitize the inactivation of TMV-RNA by artificial UV-B and UV-A light (Murphy, 1975*a*). Sulfanilamide and chlortetracycline were much more effective on a molar basis (effective at much lower concentrations) than acetone, but all three compounds appeared to act through a similar mechanism, because their effects were all partially inhibitable by O_2, a triplet quencher, and because they all produced damage which was photorepairable (see below). Hydration of uridine was not measured, but no breakage was detected when irradiated RNA was analyzed by zonal centrifugation. It is very likely that, under these conditions, cyclobutadipyrimidines also are the major photoproducts and are lethal.

In summary, it seems that there are at least two potentially lethal photoproducts that can be formed in TMV-RNA. The relative importance of these depends on solution conditions. In solutions of low ionic strength, uridine hydrates are apparently lethal. At least some dimeric photoproducts are not lethal at low ionic strength, but it is difficult to assert that for all dimers. In solutions of high ionic strength and in the presence of triplet sensitizing agents, dipyrimidines probably represent the major lethal photoproducts.

2.1.2c. Mechanism of Killing

The biological functions of TMV-RNA which are sensitive to UV and the loss of which result in "inactivation" of infectivity have not been identified. Murphy *et al.* (1973) showed that irradiation of TMV-RNA at 254 nm decreased its ability to act as a template in an *in vitro* (wheat embryo) protein-synthesizing system. However, the cross-section for loss of template activity was only ⅛ the cross section for loss of infectivity. The authors suggested several possible reasons for the increased sensitivity of infectivity relative to messenger activity: (1) TMV-RNA might have several (8) cistrons, each working independently of the others as a messenger, each equally sensitive to UV, and each essential for a successful infection; (2) infection might depend on a property of TMV-RNA, for instance, template activity for RNA synthesis, which is more sensitive to UV than is messenger activity; (3) the accuracy of translation might be more sensitive to UV than the rate, as measured by *in vitro* amino acid incorporation. Recent evidence that TMV-RNA has only three functional cistrons (Wilson *et al.*, 1978; Bruening *et al.*, 1976) argues against hypothesis 1. UV irradiation of TMV-RNA is not found to be mutagenic (Singer and Fraenkel-Conrat, 1969, 1970), making hypothesis 3 unlikely. Evidence with bacteriophages suggests that irradiated RNA is unable to serve as a template for the formation of a replicative form (Section 3.2). If this were shown to be true with TMV-RNA, it would support hypothesis 2.

The number of photochemical events, uridine hydrates and dipyrimidines, observed per inactivating biological event is 3–10 (Singer, 1971; Tao *et al.*, 1969), even though the semilogarithmic kinetics of inactivation suggest (are consistent with) a mechanism in which one quantum is required for inactivation. It may be that only one-third or fewer lesions occur at sensitive positions in the RNA; alternatively, photochemical events may cluster together and clusters may be required for inactivation (Pearson *et al.*, 1966; Johns *et al.*, 1966; McLaren and Shugar, 1964; Kleczkowski, 1960; Goddard *et al.*, 1966). The latter explanation would be consistent with a deviation in semi-logarithmic kinetics (a slowing of the rate of inactivation) sometimes observed with high doses of UV (Singer, 1971).

2.1.3. Inactivation of Whole Virus

The effects of UV radiation on the rigid-rod plant viruses are profoundly different from the effects on purified RNAs. In general, the

viruses are less sensitive to UV. The quantum yield for inactivation of whole virus is about 1% that of RNA at the longer wavelengths of the absorption spectrum (Rushizky *et al.*, 1960).

2.1.3a. Action Spectrum

Action spectra for inactivation of infectivity of TMV have been reported by Hollaender and Duggar (1936), Siegel and Norman (1958), and Rushizky *et al.* (1960) (Fig. 3). Inactivation differs for two strains of TMV, U1 and U2. U1 is the common strain of TMV. U2 was originally isolated from a preparation of U1 as an electrophoretic variant (low mobility) (Singer *et al.*, 1951) which produces an infection with "mild" symptoms. Siegel and Wildman (1954) first reported a difference in the UV sensitivities of the two strains. At 230 nm, the

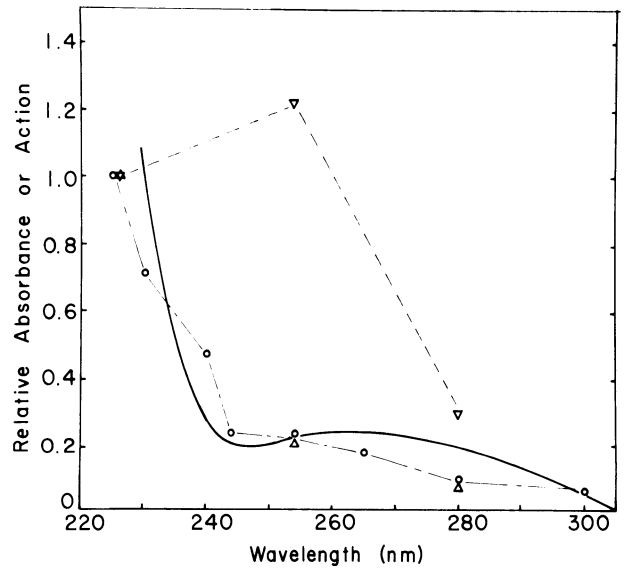


Fig. 3. Action spectra for inactivation of infectivity of TMV: O, Common strain (Hollaender and Duggar, 1936); Δ, strain U1 (Siegel and Norman, 1958); ▽, strain U2 (Siegel and Norman, 1958). All data sets normalized to 1.0 at 226 nm. ———, Absorption spectrum of TMV (T. M. Murphy, unpublished, normalized to 1.0 at 230 nm). Quantum yields for inactivation of TMV and TMV-RNA are (Rushizky *et al.*, 1960):

Wavelength	ϕ (*TMV*)	ϕ (*TMV-RNA*)
230 nm	8.7×10^{-5}	3.7×10^{-3}
248	4.0	3.4
254	4.3	3.8
265	3.9	3.5
280	1.8	3.8

sensitivities of the two strains are equal and close to that of RNA, but at the longer wavelengths, 254 and 280 nm, U1 is much less sensitive than U2 (Siegel and Norman, 1958). As one consequence, U1 would be much less sensitive to environmental (solar) UV than RNA.

2.1.3b. Protective Effect of Protein: Modification of RNA Photochemistry

The relative insensitivity of whole virus is a property of the coat protein. It does not depend on assembly of the virus particle *in vivo*, since reconstituted virus behaves like native virus (Rushizky *et al.*, 1960). Furthermore, the sensitivity of reconstituted virus depends on the strain which provides the protein, rather than the nucleic acid. Viruses reconstituted from U1 protein and U2 RNA show a sensitivity to UV identical to that of native or reconstituted U1 virus; viruses reconstituted from U2 protein and U1 RNA are as sensitive as U2 virus (Streeter and Gordon, 1967). Viruses made from U1 protein and potato virus X RNA are, like U1 virus, about fiftyfold less sensitive to absorbed UV than the corresponding free RNA at 254 nm (McCleary and Gordon, 1973).

Siegel (1957) observed that the protective effect of the protein of the U1 strain is diminished on drying. Dry U1 and dry U2 TMV had identical rates of inactivation by 254-nm radiation. The resistance of the U1 strain was recovered on rewetting of the dried virus.

The protective effect of the coat protein is an effect on the photochemistry of the enclosed RNA at, or shortly after, the time of irradiation, rather than a masking or reversal of damage that occurs at the time of infection. This is shown by the observation that the infectivity of RNA extracted from virus irradiated at 230, 254, or 280 nm is reduced by the same proportion as is the infectivity of the virus itself (Goddard *et al.*, 1966; Kleczkowski and McLaren, 1967).

The coat protein of TMV modifies the products formed in RNA by irradiation. The first indication for this came from studies of photorepair (see Section 2.1.5). The infectivity of UV-irradiated TMV-RNA is increased by illumination given after inoculation (Bawden and Kleczkowski, 1959); the infectivity of whole TMV or TRV virus is not increased by the same treatment, suggesting that there may be repairable photoproducts present in irradiated RNA but not in irradiated whole virus. Tao *et al.* (1966) showed that substitution of D_2O for H_2O did not reduce the rate of inactivation of TMV (either U1 or U2 strain)

as it did for TMV-RNA at low ionic strength, suggesting that solvent did not participate in a rate-limiting step in the inactivation of virus as it did for RNA. It is possible, however, that in this case the coat protein simply inhibited the exchange of D_2O for H_2O inside the virus near the nucleic acid. Characterization of the products of irradiation in the RNA extracted from treated virus (Tao *et al.*, 1969; Carpenter and Kleczkowski, 1969) demonstrated the formation of uridine hydrates and noncyclobutane-type dimeric forms of pyrimidines, but an absence of cyclobutadipyrimidines. The number of hydrates formed per lethal hit was about two (Tao *et al.*, 1969), higher than the number formed in RNA at low ionic strength (Small *et al.*, 1968). The number of dimeric photoproducts formed per lethal hit was about one, about equal to or slightly higher than the total number of dimers formed in RNA (0.82) but considerably higher than the number of non-cyclobutane-type dimers formed in RNA (0.27). The information from these studies is limited by the specific pyrimidine labels in the RNA used—no purine photoproducts could be detected, if indeed any were formed. However, even with this limitation, several conclusions about the effect of protein can be formed. Protein inhibits the formation of cyclobutadipyrimidines; these products are not formed even when energy is available and photosensitized reactions are occurring. Probably the protein holds the RNA in a conformation in which the formation of cyclobutadipyrimidines is not feasible (see Tikchonenko, 1975). Protein also inhibits the formation of other photoproducts by reducing the quantum yield for photoreactions—this could occur through shielding of RNA chromophores or quenching of excited states of RNA. Tikchonenko (1975) suggests that protein surrounds the bases of RNA with a hydrophobic environment, preventing solvolysis of excited bases. Although it reduces the overall rate of photoreactions, coat protein allows the formation of noncyclobutane-type dipyrimidines and of uridine hydrates. Coat protein may influence the mechanism of hydration or the source of the water in the formation of hydrates in some way that reduces the effect of D_2O on the reaction.

2.1.3c. UV Damage to Coat Protein

The coat protein of TMV may also participate in UV-induced photochemistry. At relatively low doses of UV radiation (1–30 min of irradiation, 1.5–16 lethal hit equivalents), Goddard *et al.* (1966) found that UV bound the coat protein of the U1 strain to its nucleic acid to

the extent that some subunits could not be separated from the RNA by warm sodium dodecylsulfate. Smith and Meun (1968) and Jellinek and Johns (1970) have described covalent addition of sulfhydryl groups in proteins to excited pyrimidines in DNA. However, in the case of TMV, the connection between coat protein and RNA could be broken by phenol, guanidine hydrochloride, and acetic acid: this would not be expected if the bond were formed by sulfhydryl addition to a pyrimidine. It is not known whether the linkage between coat protein and RNA is noncovalent or some unstable covalent bond.

The linkage formed between coat protein and TMV-RNA apparently does not contribute greatly to the loss of infectivity. Streeter and Gordon (1968) showed that UV radiation induced binding of U2 protein to its RNA at the same rate and to the same extent that U1 protein was bound, even though it produced lethal hits in U2 at a rate six times greater than in U1. Furthermore, a direct measurement of the efficiency of uncoating of TMV *in vivo* shows that the uncoating of irradiated virus matches that of unirradiated virus (Hayashi *et al.*, 1969).

UV causes other types of changes in TMV protein. The protein of the common strain of TMV has three tryptophan and four tyrosine residues. A study of the fluorescence properties of this protein (Magne *et al.*, 1977) indicated that irradiation at 280 nm leads specifically to indole fluorescence (maximum emission λ = 325 nm). Two of the tryptophan residues have high quantum yields ($\phi_{\text{trp }17}$ = 0.29; $\phi_{\text{trp }52}$ = 0.37) and are presumably buried. One tryptophan is strongly quenched ($\phi_{\text{trp }152}$ = 0.035), perhaps by exposure to solvent. Energy absorbed by tyrosine is efficiently transferred to tryptophan. As described above, excited tryptophan can eject an electron; the electron and the tryptophanyl radical are both highly reactive. The secondary chemistry can greatly change the protein's structure. This may explain the following observations. The low doses used by Goddard *et al.* (1966) decreased the solubility of the protein in liquid phenol. During the conventional two-phase preparation of TMV-RNA with phenol, most of the protein dissolves in the liquid phenol phase. Only a small fluffy layer of denatured viral protein appears at the phenol–water interface. When the virus used is irradiated, a very large fluffy layer appears at the interface, and separation of the two phases is difficult. The amount of protein apparent at the interface suggests that a large number of subunits are altered per lethal hit. However, Goddard *et al.* (1966) did not quantitate this alteration in phenol solubility. Higher doses of UV have been shown to make TMV more labile to heat (Kleczkowski, 1954; Oster

and McLaren, 1950), to promote the dissociation of protein from the virus particles as observed by electron microscopy (McLaren and Kleczkowski, 1967), to cause the loss of antigenic activity (Kleczkowski, 1962), and finally to disrupt the particle and allow the release of the (highly altered) nucleic acid (Zech, 1961).

2.1.4. Inactivation of Virus *in Vivo*

Ultraviolet light can inactivate TMV within host cells. Siegel and his coworkers have tested the degree of inactivation of infective centers as a function of time by inoculating local lesion hosts (*Nicotiana glutinosa*), waiting for a measured interval, then directly irradiating the inoculated epidermis. In general, the survival of viral infective centers (potential lesions) remained constant for a period of time ranging from one to several hours; then it increased rapidly to a new level; then it again remained fairly constant (Siegel and Wildman, 1956; Siegel *et al.*, 1957) (Fig. 4). U2 infective centers showed two periods during which survival increased.

The objective of these studies was to characterize early stages of the infective process. The time required before the increase in survival was related, at least in part, to the uncoating of the virus, as shown by the difference in time required by U1 or U2 RNA (1 hr) compared to U2 whole virus (2 hr) and U1 whole virus (6 hr). The reason for the decrease in sensitivity was ascribed by Siegel and Wildman (1956) to the synthesis of new virions; these authors found single-hit inactivation

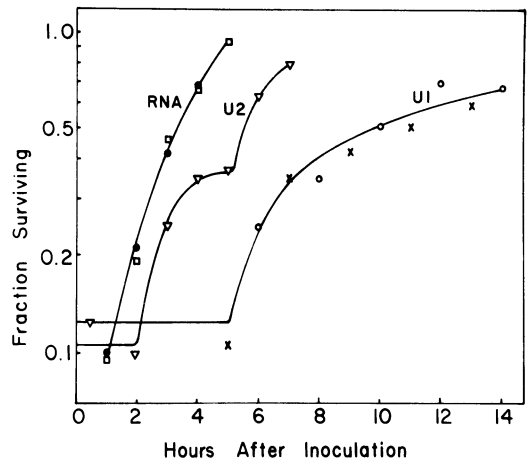


Fig. 4. Survival of infective centers produced on leaves of *Nicotiana glutinosa* by TMV-RNA (□, ●), TMV (strain U2, ▽), and TMV (strain U1, ×, ○), plotted as a function of time of irradiation of inoculated leaves. Dose of UV: intact U2 and RNA, 90 sec: U1, 5 min. Redrawn from Siegel *et al.* (1957).

kinetics in the early, sensitive stage and multiple-hit kinetics in the later, less sensitive stage. Bawden and Kleczkowski (1960) criticized the methods, data, and interpretations of the inactivation-kinetics experiments, but they did not dispute the conclusions.

2.1.5. Repair of UV-Induced Inactivation

2.1.5a. Photoreactivation of TMV-RNA

A light-dependent repair of UV radiation-induced loss of infectivity of plant viruses was discovered by Bawden and Kleczkowski (1953, 1955). The repair was not found with irradiated rigid-rod viruses such as TMV and TRV (Bawden and Kleczkowski, 1955; Harrison and Nixon, 1959), but was shown to occur with irradiated free RNA extracted from unirradiated virus (Bawden and Kleczkowski, 1959). Since that time, a majority of the studies on the chemistry and mechanism of photorepair of RNA and on its physiological implications have used TMV-RNA as a model system.

The light-dependent repair, photorepair or photoreactivation, is observed by measuring the relative infectivities (numbers of lesions per leaf on a local lesion host) of unirradiated and UV-irradiated TMV-RNA, both on plants kept in the dark for several hours after inoculation and on plants illuminated immediately after inoculation (Fig. 5).

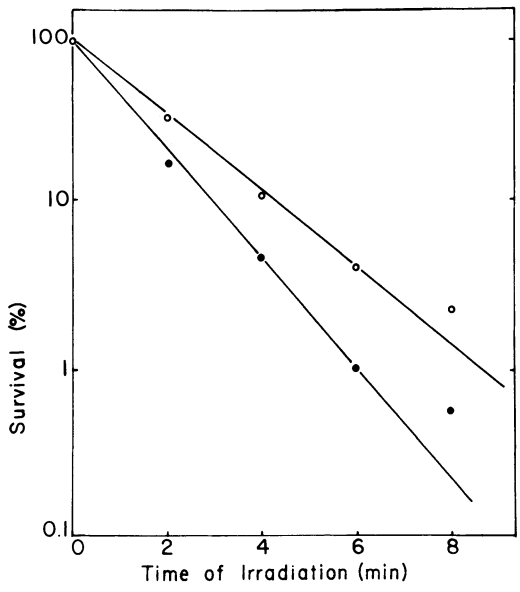


Fig. 5. Photoreactivation of TMV-RNA irradiated at 254 nm. Solutions of TMV-RNA were irradiated in aqueous solution at 50 μg/ml, then assayed on leaves of *N. tabacum* var. *Xanthi* nc. After inoculation, plants were either placed immediately in light (○) or held in dark for 8 hr (●). Redrawn from Merriam and Gordon (1965).

The degree of photorepair or "photoreactivated sector," f_p, is defined as

$$1 - \frac{\text{dose to reach a given loss of infectivity in the dark}}{\text{dose to reach the same loss of infectivity in the light}}$$

Under conditions where inactivation follows single-hit kinetics (low doses of UV), a single dose of UV may be used, and f_p can be measured by the formula (Werbin *et al.*, 1967)

$$1 - \frac{\log y \text{ in light}}{\log y \text{ in dark}}$$

where y is the surviving fraction of infectivity. The f_p can be thought of as the fraction of the UV damage that is repaired by exposure to light.

The light-dependent increase in number of lesions occurs only with UV-irradiated RNA and not with control RNA. Although the formula for calculating f_p takes into account possible effects of light on the infectivity of unirradiated RNA, in practice, light- and dark-treated plants produce the same number of lesions when inoculated with the same unirradiated sample of TMV-RNA. The UV specificity of the effect provides the basis for the names *photorepair* and *photoreactivation*. Observations of a light-dependent increase in the infectivity of UV-irradiated RNA when the light is applied *in vitro* (see below) support this interpretation. However, there has been no chemical characterization of repaired TMV-RNA, so the possibility that light supplied to inoculated host plants makes these plants more permissive for the growth of UV-irradiated RNA cannot be ignored.

In general, photorepair of TMV-RNA depends on the host. Different species of plants repair irradiated TMV-RNA to different extents. Werbin *et al.* (1966) found that the photorepair of a given sample of irradiated TMV-RNA was greater on leaves of *Nicotiana tabacum* var. *Xanthi* nc. than on the primary leaves of *Phaseolus vulgaris* L. var. *pinto* or on *Chenopodium amaranticolor* Coste and Reyn. Murphy and Gordon (1971*a*) discovered that when *N. tabacum* var. *Xanthi* nc. plants were kept in the dark, their ability to photorepair irradiated TMV-RNA progressively decreased. The f_p declined from 0.25 (light-grown plants) to 0.1 after 4 days in the dark, and to 0.05 after 8 days in the dark. The plants recovered their ability for photorepair 12 hr after they were returned to the light. White, blue, and green fluorescent lights promoted the ability of the plant for repair; red

and near-UV lamps were not effective. The colors effective in promoting ability for photorepair were not the same as those involved in the repair process.

Irradiated TMV-RNA is maximally photoreactivable on *N. tabacum* var. *Xanthi* nc. immediately after it is inoculated on a leaf. Illumination of the leaf itself before inoculation has no effect. Immediately after inoculation, illumination of the leaf with twenty 40-W cool-white fluorescent tubes beginning about 15 sec to 1 min after inoculation and continuing for a period as short as 2 min gives maximal photoreactivation (f_p = 0.25; Murphy and Gordon, 1971*a*). If, after inoculation, the leaves are held in the dark for as little as 1 hr, the effect of subsequent light treatments is decreased. After 5 hr in the dark, a light treatment has no effect (Merriam and Gordon, 1965).

The action spectrum of the repair reaction shows that near-UV light is most effective. In pinto beans, the peak of effectiveness comes at 366 nm; there is a small effect from blue light. In *Xanthi* tobacco, the maximum photorepair is also in the UV-A, but blue light is more effective than in beans (Hidalgo-Salvatierra and McLaren, 1969; Murphy and Gordon, 1971*b*).

2.1.5b. Effect of TMV Protein on Photoreactivation

TMV protein inhibits photorepair in two different ways. First, the encapsulation prevents the formation of photorepairable lesions in the RNA. As mentioned above, irradiated TMV and TRV virus particles cannot be photorepaired (Bawden and Kleczkowski, 1955; Harrison and Nixon, 1959). Furthermore, RNA extracted from irradiated TMV cannot be photorepaired (Bawden and Kleczkowski, 1959). This effect may well reflect the chemistry of photoinactivation, since there are differences between the number and nature of photoproducts in RNA from irradiated TMV and in TMV-RNA irradiated free of protein (Small *et al.*, 1968; Tao *et al.*, 1969). Second, encapsulation *in vitro* prevents the photorepair of TMV-RNA which has been irradiated in its free state and which would be photorepairable if used free to infect a host (Small and Gordon, 1967). Encapsulation does not repair the photorepairable damage or render it unrepairable chemically, since the RNA extracted from the encapsulated virus can once again be repaired. It is likely that this second effect reflects a change in the relative timing of repair and RNase degradation of the irradiated genome; the slow

uncoating of the virus (see Fig. 4) may favor degradation over repair, translation, and replication.

2.1.5c. Biochemical Basis of Photoreactivation

The chemical nature of the lesions that are subject to photorepair has been a matter of considerable experimentation and discussion. Initial experiments failed to show a good correlation between f_p or $1-f_p$ and the number of dimers per hit when TMV-RNA was irradiated under different conditions of wavelength, temperature, and ionic strength (correlation coefficient ± 0.2, number of conditions 8; Merriam and Gordon, 1967). In a comparison of irradiation in H_2O and D_2O, the number of dipyrimidines per lethal hit doubled, while the f_p and the number of uridine hydrates per lethal hit remained constant (Small *et al.*, 1968), a finding that could be interpreted as implying that hydrates were involved in photorepair. In a similar experiment comparing irradiation in the absence and presence of Mg^{2+}, the number of dipyrimidines per lethal hit increased, while the number of hydrates per lethal hit and the f_p remained relatively constant (Singer, 1971; Kirwan, Singer, and McLaren, quoted in Gordon *et al.*, 1976).

There are several lines of evidence available now that weigh against hydrates and in favor of cyclobutadipyrimidines as photorepairable lesions. Evans and McLaren (1968) irradiated TMV-RNA in a concentration of HCN that reduced photohydration of poly(rU) to a negligible level, while reducing dimerization by only 50%; the f_p decreased by only 40%. The analysis of photoproducts in RNA extracted from irradiated TMV (nonphotoreactivable) showed that hydrates were still present, but that cyclobutadipyrimidines were absent (Tao *et al.*, 1969; Carpenter and Kleczkowski, 1969). Cyclobutadipyrimidines were present in irradiated TMV-RNA (photoreactivable) (Small *et al.*, 1968), and this is the major recognized chemical difference between the two preparations of irradiated RNA. Finally, the irradiation of TMV-RNA (and PVX-RNA) in the presence of acetone leads to the formation of cyclobutadipyrimidines as the only detectable pyrimidine photoproduct, and these RNAs are photoreactivable (Huang and Gordon, 1972; Murphy, 1975*a*).

It seems clear that the presence of cyclobutadipyrimidines is sufficient for photorepair to occur, and that the presence of uridine hydrates is not sufficient. This probably means that photorepair breaks the

cyclobutane link between cyclobutadipyrimidines, a situation analogous to photorepair of UV-irradiated DNA, but this conclusion will remain uncertain until it is possible to analyze repaired RNA chemically.

There is some information about other aspects of the mechanism of photorepair of TMV-RNA. The action spectrum of the repair reaction does not resemble the action spectrum for photosynthesis. Additional evidence that photorepair is not dependent on photosynthetic components comes from the observation that white sectors of a variegated mutant of *Xanthi* tobacco, sectors with only 2% of the normal chlorophyll content, show normal photorepair of UV-irradiated TMV-RNA (McLaren *et al.*, 1970). The dependence of photorepair on the species and condition of the host and the timing requirements for maximal photorepair cited above suggest that a leaf cell component sensitizes a fraction of the UV-induced damage in TMV-RNA to photoreactions that remove or in some other way ameliorate the damage.

A demonstration of photorepair of UV-irradiated TMV-RNA *in vitro* using a partially purified extract of *N. tabacum* var. *Xanthi* nc. leaves (Gordon *et al.*, 1971; Hurter *et al.*, 1974) supports the idea that RNA photorepair is a direct reversal of UV-induced lesions. Extracts were concentrated by ammonium sulfate precipitation and subjected to gel filtration. The resulting solution had little RNase activity, and yeast RNA in excess was added to protect the TMV-RNA from inactivation by RNase. The mixture still inactivated TMV-RNA to a level of 20% of the controls, but the final level was stable and could be accounted for in further manipulations. The illumination of a mixture of extract and UV-irradiated TMV-RNA with 365-nm light caused a fourfold increase in the infectivity of the TMV-RNA relative to unilluminated controls. The activity of the extract was destroyed by boiling, lost during storage, and occasionally (30% of experiments) not found at all. The apparent lability of the activity is suggestive of an enzyme, but further confirmation is needed. The finding that indole compounds can sensitize the light-dependent splitting of cyclobutadiuracil (Heléne and Charlier, 1971; Chen *et al.*, 1976) suggests that the activity in leaf extracts might be due to such compounds. The compounds might be part of the active site of a photorepair enzyme, but might be working in a more nonspecific manner.

2.1.5d. Physiological Significance of RNA Photorepair

The significance of RNA photorepair to the plant is still a mystery. One hypothesis, formed by analogy with DNA photorepair,

suggests that an RNA photorepair enzyme protects plant RNA from damage by sunlight UV. DNA photorepair is mediated by a single enzyme which, in the dark, binds to cyclobutadipyrimidines and, in the light, splits the cyclobutane ring and falls off the DNA (Setlow, 1968). In bacteria, the same enzyme repairs cyclobutadipyrimidines in host DNA and in the DNA of infecting bacteriophages; cyclobutadipyrimidines in host and viral DNA will compete in binding the enzyme (Harm *et al.*, 1968; Lennox *et al.*, 1954; Metzger, 1963). The irradiation of leaves of *N. tabacum* var *Xanthi* nc. at 254 nm inhibited the photorepair of UV-irradiated TMV-RNA that was subsequently applied (Murphy and Gordon, 1971*b*). The inhibition was stable in the dark but reversed by illumination. The colors of light most effective in photorepair of TMV-RNA were most effective in reversing the inhibition. The results were consistent with the hypothesis that the enzyme that repairs UV-damaged TMV-RNA interacts also with UV damage to cellular nucleic acids. However, other explanations are possible. For instance, UV light could destroy an enzyme or a nonenzymatic compound that is required for photoreactivation of TMV-RNA. Visible light could induce the resynthesis or repair of this factor. The fact that the colors of light effective in such resynthesis or repair are the same as those effective in TMV-RNA photoreactivation could be coincidence, and the fact that these colors are different from the colors promoting photoreactivation ability after a dark treatment (Murphy and Gordon, 1971*a*) could simply mean that the resynthesis or repair processes are different in the two situations.

Attempts to show more directly a light-dependent repair of a plant function dependent on RNA have not been successful. Wright and Murphy (1975) found no photorepair of the UV-induced inhibition of amino acid incorporation by cultured tobacco cells. A photorepair of nitrate reductase synthesis was observed, but could best be explained by repair of UV damage to DNA-dependent mRNA synthesis. Diner and Murphy (1973) showed that the loss of ability to photorepair UV-irradiated TMV-RNA that was achieved by putting leaves of *N. tabacum* var *Xanthi* nc. in the dark for 8 days did not represent a corresponding loss of ability to photorepair visible UV damage to the leaf cells themselves ("bronzing," epidermal necrosis). At present, there is no evidence that the ability to photorepair viral RNA has any significance in the protection of plant cell components from UV damage. It is possible that such protection occurs, but has not been detected. It is possible that viral RNA photorepair is fortuitous, dependent on plant components (e.g., tryptophan, indole acetic acid) with some unrelated

function. It is possible that RNA viruses, and thus viral RNA repair, have some unrecognized function important to the metabolism or genetics of healthy cells.

2.2. Flexuous-Rod Viruses: PVX

2.2.1. General

Potato virus X (PVX), clover yellow mosaic virus, and cabbage black ringspot virus represent a family of plant viruses which are longer and thinner than the rigid-rod viruses like TMV, and which appear slightly curved in electron micrographs. PVX is a hollow cylinder, 580 nm long by 13 nm in diameter, with a 3.4-nm diameter central channel. As in TMV, the protein subunits of PVX are arranged in a helix, but there are nine to ten protein subunits per turn of the helix and four nucleotides per protein subunit spaced at 5.5-nm intervals. The pitch of the helix is 3.3–3.6 nm. In his article on nucleic acid structure, Tikchonenko (1975) noted the differences between PVX and TMV (length, diameter, pitch, spacing of nucleotides), but emphasized their similarities (hollow cylindrical shape, buried RNA, a highly ordered secondary structure of RNA extending throughout the particle). Nevertheless, the differences in size and shape between PVX and TMV have suggested to many workers that there are different relationships between the capsid proteins and the RNA. The photochemical inactivation of PVX has been studied quite extensively, in part to help determine the nature of the PVX capsid protein–RNA interaction.

2.2.2. Inactivation

The action spectrum for inactivation of PVX infectivity is similar (although not identical) to the absorption spectrum of PVX-RNA (Kleczkowski and Govier, 1969) (Fig. 6). Quantum yields for inactivation of RNA and whole virus vary with wavelength. At 260 nm, the values are 1×10^{-3} for RNA and 0.62×10^{-3} for the virus. This difference is not great, especially compared to the behavior of TMV (strain U1). Inactivation of a "phenotypic hybrid" virus made by reconstitution of TMV protein with PVX-RNA confirms that the dif-

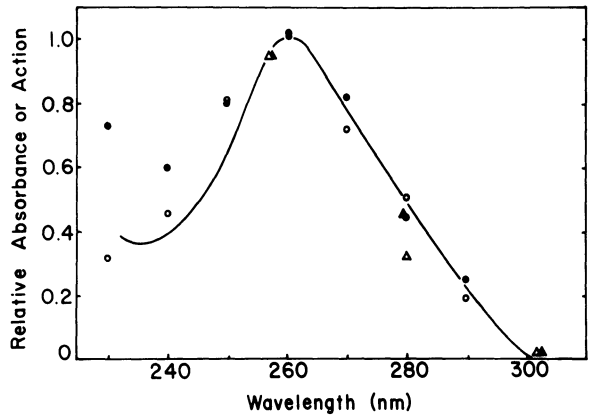


Fig. 6. Action spectra for inactivation of infectivity of PVX (●, ▲) and PVX-RNA (○, △). Data from Kleczkowski and Govier (1969) (●) and Govier and Kleczkowski (1970) (○) normalized to 1.0 at 260 nm; data from Breck (1971) (▲, △) normalized to 0.95 at 254 nm. ———, Absorption spectrum of PVX-RNA (Breck, 1971). 026

ference between quantum yields of inactivation of TMV and PVX relates specifically to the protein: PVX capsid protein is less protective.

Other observations support the idea that the RNA in PVX virus reacts photochemically more like RNA in free solution than like RNA inside TMV viral particles. The rate of inactivation of PVX depends on the isotopic composition of the solvent, being greater in H_2O than in D_2O (Breck, 1971; McCleary and Gordon, 1973). This observation is similar to that found with TMV-RNA and PVX-RNA irradiated free in solution and in contrast to that found with TMV and the hybrid virus containing TMV protein and PVX-RNA. Cyclobutadipyrimidine photoproducts can be found in RNA extracted from UV-irradiated PVX (Huang and Gordon, 1974), and UV-irradiated PVX can be photoreactivated. These observations, too, are characteristic of the irradiation of free RNA. In a related study, Kust *et al.* (1972) showed that the RNA in PVX reacts completely with 1.5% formaldehyde at room temperature in 24 hr, while the RNA in TMV shows no reaction under these conditions.

It would not be accurate to say that encapsulated PVX-RNA reacts exactly the same as free RNA. The magnitude of the isotope effect was less for PVX than for PVX-RNA. For instance, at 254 nm, the ratios of the rates of inactivation in H_2O and D_2O were 1.17 ± 0.11 for PVX and 1.90 ± 0.50 for PVX-RNA. This shows that PVX protein does affect the mechanism of photochemical inactivation of PVX-

RNA. It would be useful to know whether isomers of cyclobutadipyrimidines formed in irradiated PVX are the same as those formed in irradiated free PVX-RNA. However, there are as yet no more details available concerning the chemistry or biochemistry of inactivation of this virus.

2.2.3. Repair

PVX, clover yellow mosaic virus, and cabbage black ringspot virus all can be photoreactivated by their local lesion hosts (Bawden and Kleczkowski, 1955; Chessin, 1965). The degree of maximum photoreactivation of PVX is approximately the same as that of PVX-RNA and TMV-RNA (McCleary and Gordon, 1973). Kleczkowski and Govier (1969) found that the degree of photoreactivation of PVX depended on the wavelength of UV with which the virus was irradiated, but McCleary and Gordon (1973) did not confirm this: the lack of agreement may reflect the differences in the assay plants and in the conditions of photoreactivation.

The responses of the flexuous-rod viruses to UV inactivation and photoreactivation confirm the hypothesis that the packaging of viral RNA within the capsid proteins is very different from the packaging seen in TMV. The higher inactivation cross-sections and quantum yields of PVX relative to TMV suggest that the PVX protein neither shields nor quenches RNA photoreactions as much as TMV protein. The presence of cyclobutadipyrimidines in UV-irradiated PVX may mean that the RNA bases in this virus are not rigidly constrained in a way that prevents the juxtaposition of adjacent pyrimidines. Photoreactivation of irradiated PVX probably reflects the presence of cyclobutadipyrimidines and an uncoating process in the infected cell that allows the operation of the photoreactivation system before the RNA is degraded by nucleases. These observations, other observations concerning the interactions of the RNA within the virus with the solvent, and the appearance of the virus in the electron microscope all are consistent with a model in which PVX capsid proteins are more loosely spaced around or along the RNA genome than are TMV capsid proteins. Alternatively, the differences between the photochemistry, chemical reactivity, and morphology of PVX and TMV may depend more on specific bonds formed (or not formed) between the nucleic acid bases and the protein subunits and less on the steric effects produced by tight (or loose) packaging (Tikchonenko, 1975).

2.3. Polyhedral and Other Viruses

2.3.1. General

The polyhedral plant viruses, including tomato bushy stunt virus, Rothamsted tobacco necrosis virus, tobacco ringspot virus, cucumber mosaic virus, and southern bean mosaic virus, probably represent the largest group of plant RNA viruses. These viruses are icosahedrons formed from RNA and 180 protein subunits. Protein subunits of some viruses can form empty capsids; subunits of other viruses require the RNA. When present, the RNA occupies the central chamber. When the RNA is small (10^6 daltons), most of it lies in the outer part of the chamber, close to the proteins; when it is larger (2.4×10^6 daltons), more RNA is found in the very center (Fischbach and Anderegg, 1976). In contrast to the rigid-rod and filamentous viruses discussed above, the RNA in polyhedral viruses is generally considered to be structurally similar to RNA in solution. Evidence from hyperchromicity and circular dicroism, for instance, suggests that double-stranded regions involve 73% and 70%, respectively, of wild cucumber mosaic virus RNA in capsids and in solution (Tikchonenko, 1975).

There is some information on the photobiology of the viruses cited above, although not as much as for TMV or PVX. In some cases, the photobiology of these viruses has been studied to ascertain the effect of encapsulation on the structure or environment of the RNA inside. Many of these viruses are multicomponent viruses: in certain cases, UV radiation has been used to distinguish between the chemical and biological properties of the different components. However, much less has been done than with corresponding animal virus systems, and the potential for further work is great.

2.3.2. Effect of Encapsulation on Inactivation and Repair of Polyhedral Viruses

The initial report of Bawden and Kleczkowski (1955) indicated that polyhedral viruses (cucumber mosaic, tobacco ringspot, cabbage black ringspot viruses) were more sensitive to inactivation by UV radiation than TMV or even PVX. These viruses, tomato bushy stunt virus, and tobacco necrosis virus (TNV) could be photoreactivated. Further work showed that not only were the inactivation cross-sections of TNV and TNV-RNA similar, but their action spectra were identical between

230 and 290 nm, and these action spectra matched the absorption spectrum of free RNA (Kassanis and Kleczkowski, 1965). A hybrid virus formed from TMV (U1)-RNA and the protein of cowpea chlorotic mottle virus showed the same sensitivity to UV as the free RNA. This virus could be photoreactivated on *N. tabacum* var. *Xanthi* nc. (Grouse *et al.*, 1970). Thus encapsulation of RNA into polyhedral plant viruses does not seem to change the photochemical reactivity of the RNA.

There is an interesting amount of structural detail available for southern bean mosaic virus (SBMV). As with other polyhedral viruses, there is no significant difference between the inactivation cross-sections of virus and viral RNA and no difference between values of f_p observed with the two infectious agents (Price, 1965; Sehgal, 1973). These results can be related to structural models for SBMV derived from X-ray crystallography at 5 Å resolution (Suck *et al.*, 1978). Of the 36% of the RNA that could be discerned in the crystallographs as ordered phosphate residues, most was separated from the protein shell by a space of several angstroms. The authors suggested that the bases attached to these phosphates extended out toward the capsids and into the virus interior. Presumably, these bases were less ordered or less symmetrically ordered, since they could not be resolved. The amount of space between phosphates and capsid proteins suggested the presence of some solvent. Sixty-four percent of the RNA could not be resolved; it was probably in the virus interior and was either disordered or was not icosahedrally symmetrical. While the data are not yet complete, the degree of disorder in the bases and the potential volume for solvent in the virus interior help explain why encapsulation of SBMV RNA does not greatly limit the photochemical reactivity of the RNA.

Fluorescence emission studies (Herzog *et al.*, 1977) also point to a lack of interaction between RNA and protein in brome mosaic virus. Excitation of the protein dimers at 280 nm leads to fluorescence of tyrosine with an unusually high quantum yield (ϕ = 0.07) and fluorescence of tryptophan with an unusually low quantum yield (ϕ = 0.056, 0.014 for the two tryptophans). Formation of capsids further quenches the fluorescence of the low-yielding tryptophan. Addition of the RNA, however, does not affect the fluorescence: that is, virus and empty capsids show the same spectrum. Thus the nucleic acid bases are probably well separated from the tyrosines and tryptophans of the capsid subunits. Although the characteristics of UV-induced inactivation and photoreactivation of brome mosaic virus have not yet been reported, we would predict that they would match the responses of brome mosaic virus RNA.

2.3.3. Dark Repair of TNV

Kleczkowski (1968) has reported observations suggesting "dark reactivation" of lethal UV-induced damage in tobacco necrosis virus. Preparations of control and irradiated virus were assayed on French bean, tobacco, and *Chenopodium amaranticolor* with and without the application of photoreactivating light. The assays using French bean and tobacco gave identical results: inactivation cross-sections for the dark-treated assays were the same, and there was the same degree of photoreactivation. In sharp contrast, the assay using *C. amaranticolor* gave inactivation cross-sections for dark- and light-treated hosts which were equal to each other (no photoreactivation) and were equal to the cross-sections for light-treated assays with bean and tobacco. The author concluded that a fraction of damage which was lethal in bean and tobacco in the dark was repaired in *C. amaranticolor* in the dark and that this damage was the same as that which was photorepaired by beans and tobacco. The putative dark repair system may be specific not only for *C. amaranticolor* but for TNV, since Werbin *et al.* (1966), assaying UV-irradiated TMV-RNA in the same three species, found full photorepair and no evidence for any dark repair in all cases. The report by Kleczkowski (1968) is the only well-controlled observation of dark repair of viral RNA, and as such deserves confirmation and further study.

2.3.4. Multicomponent and Complex Viruses

The UV irradiation of the bottom component of raspberry ringspot virus leads to the formation of dimers of the smaller of two RNA components (Mayo *et al.*, 1973). This shows that the two RNA molecules are in the same particle. The chemical nature of the linkage has not yet been determined.

Lettuce necrotic yellows virus is a complex lipid-containing virus possibly analogous in structure to animal viruses like vesicular stomatitis virus. It has been reported to be about eight-fold more sensitive to 254-nm radiation than the U2 strain of TMV (McLean and Crowley, 1969). Since U2 TMV is about 6 times more sensitive than U1, and U1 has about 2% of the quantum yield of free TMV-RNA, it may be that the UV sensitivity of lettuce necrotic yellows virus is the same as that of its free RNA. However, this point should be checked directly.

Observations by Wright and Murphy (1978) and Murphy *et al.* (1978) emphasize the role of membranes in the UV-induced killing of

plant cells, even at relatively low doses. There might be a similar effect on a lipid-containing plant virus. Such a virus could serve as a valuable tool in further studies along these lines.

2.3.5. Inactivation *in Vivo*

The sensitivities of viral infective centers to irradiation of the inoculated leaf may reveal information concerning the timing and mechanics of the infection process. Bawden and Harrison (1955) tested TNV on *Phaseolus vulgaris*, and McLean and Crowley (1969) tested lettuce necrotic yellows virus on *N. glutinosa*. The sensitivities of local lesion formation by these viruses showed changes substantially similar to those seen with TMV (Section 2.1.4): sensitivity was constant for several hours, then decreased, then remained constant for several hours. The similarities are rather surprising, since the structures of the three viruses are so different. However, the changes in sensitivity may not reflect the same process for the different viruses. Siegel and Wildman (1956) reported multiple-hit kinetics of inactivation of TMV after the decrease in sensitivity; neither Bawden and Harrison (1955) nor McLean and Crowley (1969) obtained such results.

2.4. Viroids

The subject of viroids is reviewed extensively in Vol. 11 of this series (Diener and Hadidi, 1977). The kinetics of inactivation of potato spindle tuber viroid (PSTV) by UV radiation served to throw some light on the properties of these agents. The rate of inactivation of the infectivity of PSTV was much slower than that of tobacco ring spot virus; inactivation required about 70–90 times the dose as tobacco ring spot virus. The dose was about comparable to that required to inactivate the satellite of tobacco ring spot virus which has a molecular weight of $7.5–8.5 \times 10^4$ (Diener *et al.*, 1974). The dosage and the first-order kinetics of inactivation indicated that the infectious entity was a single, small RNA molecule rather than a multicomponent agent which held information necessary for successful infectivity in several RNA species. The behavior of PSTV was consistent with the formulation of the viroid as a single low-molecular-weight RNA with a molecular weight of about 10^5.

3. RNA BACTERIOPHAGES

3.1. General

The composition of RNA bacteriophages is discussed in earlier chapters of this series (Eoyang and August, 1974; Fiers, 1979). Briefly, each particle consists of a spherical or polyhedral capsule composed principally of one type of protein, with small numbers of one or two other proteins. The genome is a single-stranded RNA molecule of about 1 to 1.5 $\times$ 10^6 molecular weight. These particles specifically infect male bacterial cells by absorption to pili of the host, penetration of the RNA into the host cell, synthesis of phage-coded coat and synthetase proteins, synthesis of new phage RNA via a replicative form, synthesis of maturation proteins, packaging, and ultimate exit of the phage from the cell (Weissmann, 1974).

The overall parameters of the UV inactivation of a number of RNA phages including fr, f2, R17, MS2, and Qβ are given in a paper by Rauth (1965) and in a review by Gordon *et al.* (1976). The action spectra of these viruses closely resemble the absorption spectra of the RNA, and the quantum yields of inactivation of the intact viruses and free RNA are of the same order of magnitude, about 1 $\times$ 10^{-3}.

3.2. Effect of UV on the Infectious Cycle of RNA Phages

The effect of UV irradiation on the infectious cycle of f2 and MS2 phages has been studied by Werbin *et al.* (1967, 1968) and Yamada *et al.* (1973). Werbin and co-workers found that the initial reversible adsorption of the phages to the pili of the host cell was not affected by UV. In contrast, the penetration of phage RNA was reduced by UV, but to a lesser extent than the infectivity. UV doses that reduced phage survival to 10% and 1% reduced the penetration of RNA to 45% and 10%, respectively, of control values. Yamada *et al.* found that, after injection into the host cell, RNA from control phage is stable even when protein synthesis is prevented by chloramphenicol. After synthesis of RNA synthetase, part of the RNA is converted to a replicative form which is detected by stability to RNase subsequent to phenol extraction. The injected RNA from phage irradiated to 10% survival was broken down to a mixture of low-molecular-weight fragments in the host cell, and there was no evidence for the formation of replicative forms. In spite of the failure of RNA from irradiated phage to form replicative forms,

this RNA did induce the formation of RNA synthetase at levels approaching 30% of control values. These results can be explained by the following scenario. Some of the UV-damaged phages still are able to insert RNA into host cells. The damaged RNA in many of the cells is fragmented by host nucleases, which appear to degrade UV-damaged phage RNA while unirradiated RNA is stable. Some of the UV-damaged RNA nevertheless serves as a messenger for the synthesis of phage RNA synthetase; however, the synthetase cannot utilize the UV-damaged phage RNA as a template, and the infection thus aborts.

3.3. Molecular Basis for the Action of UV on RNA Phages

The inactivation of R17 phage and its free RNA has also been examined by Cerutti and his coworkers on a molecular basis (Cerutti *et al.*, 1969; Remsen *et al.*, 1970). When the intact phage was irradiated (Remsen *et al.*, 1971), the capsomer proteins appeared to restrain the RNA in such a manner that cyclobutadipyrimidines were not formed, in exact analogy to the behavior of the U1 strain of TMV. The logarithm of the survival of plaque-forming activity on irradiation at 280 nm was a linear function of the number of uridine hydrates formed, with 0.75 uridine hydrates detected per lethal hit. The RNA extracted from the phage irradiated under these conditions was used as a messenger *in vitro*. It was found that, on average, five times more uridine hydrate molecules are needed to decrease the overall messenger activity to 37% than to decrease the infectivity to the same extent. It required eleven times more uridine hydrate molecules to reduce the synthesis of synthetase to 37% of control levels.

These results corroborate the above findings of Yamada *et al.* (1973) that extensively irradiated phage is still able to form synthetase molecules *in vivo*, and lend credence to the hypothesis that the basis of the loss of infectivity is the inability of the extensively irradiated RNA to serve as a template. This may explain the observation of Murphy *et al.* (1973) that the UV sensitivity of the infectivity of TMV-RNA is 8 times greater than the sensitivity of the translational activity.

3.4. Photoreactivation of RNA

There is some question as to whether the RNA phages or the free phage RNA can be photoreactivated. Zinder (1965), Rauth (1965), and

Winkler (1964) reported finding no photoreactivation after irradiation of the intact RNA phages. Werbin and coworkers (1967) irradiated free f2 RNA at 254 nm and found a slight degree of photoreactivation. In protoplasts of an F^- strain of *E. coli* K12, quantum yields of 2.7×10^{-3} and 3.3×10^{-3} in the light and dark, respectively, were observed. In our laboratory, F. J. Ryan was unable to demonstrate experimentally significant photoreactivation of Qβ-RNA using similar techniques.

3.5. Variants of Qβ Bacteriophage

There are a number of variant RNA molecules which are formed when a Qβ replicase reaction is run without added template (Kacian *et al.*, 1972). Both of the complementary strands of the variants are recognized by Qβ replicase so that the synthesis of these molecules is autocatalytic. One strand is usually made in excess and is designated as the (+) strand. The use of one of these molecules, MDV-1, in UV studies is attendant with several advantages. The molecule is small, and, in the case of MDV-1, the sequence is known, so that it is possible to determine the exact sites of UV lesions. Synchronous replication of the molecule *in vitro* is readily accomplished, and highly labeled molecules may be readily obtained. Finally, in the *in vitro* situation, the sole function of the MDV-1 molecule is replication. It is thus possible to study the effects of UV on this function unimpeded by the necessity of the molecule to act as a messenger or to be packaged within a phage head. The action of UV on the replication of MDV-1 has been studied in a series of papers by Ryan and his associates (Ryan, 1976; Ryan *et al.*, 1977, 1979). The quantum yield of loss of template activity was determined by two independent procedures. The first, termed the "kinetic assay," measured the rate of RNA synthesis under conditions where the RNA template was rate-determining. This procedure gave a value of $\phi = 1.1 \times 10^{-3}$. The second assay, termed the "involvement assay," utilized synchronous conversion of single-stranded template molecules to double-stranded structures under conditions of low temperature such that further replication no longer occurred. Under these conditions, UV irradiation of the (+) strand prevented the formation of full-length, double-stranded RNA as determined by gel electrophoresis following the deproteinization of the reaction mixture. The quantum yield was a measure of the loss of the conversion of single- to double-stranded DNA and was found to be 1.6×10^{-3}. The agreement between these two determination of quantum yield is considered to be

good in view of the completely different parameters which are determined. When gel electropherograms were run of the replicase mixtures, molecules intermediate in size between single-stranded (+) strands and full-sized double-stranded structures were noted. The intermediate-sized structures were especially pronounced in reaction mixtures using irradiated (+) strands. These intermediate-sized structures were shown to consist of a full-sized single-stranded template annealed to a partially completed, newly synthesized strand. Small amounts of the intermediate-sized structures were also found in replicase reaction mixtures in which the unirradiated (+) strand was used as a template. It was hypothesized that the MDV-1 + strand contains regions (loops?) which inhibit the replicase, and inhibition by these loops is increased by lesions induced by UV.

The chemical nature of the lesions induced by the 254-nm radiation was determined (Ryan *et al.*, 1977). It was found that uridine hydrate production follows first-order kinetics to about 1% survival of nonhydrated strands, assuming random distribution of photoproducts. About 1.4 uridine hydrates were found per strand. The introduction of pyrimidine hydrates into an RNA molecule thus appears to block the ability of the molecule to function as a template for RNA replicase. Cyclobutadipyrimidines did not seem too important in the inactivation of MDV-1. The molecule had to be irradiated to the level of eight lethal hits before one dimer was formed.

4. ANIMAL VIRUSES

4.1. Vesicular Stomatitus Virus

4.1.1. General

Vesicular stomatitus virus is a well studied, bullet-shaped animal virus. It is relatively easy to handle and prepare in large amounts. Accordingly, this virus has served as a prototype for investigations dealing with many aspects of animal virus behavior. The properties of VSV are discussed in extensive detail in Volume 4 of this series (Wagner, 1975). The virion is a single-stranded, negative-strand virus which contains the following proteins: L, the transcriptase; G, a glycoprotein found in the virus envelope; M, a matrix protein found near the virus envelope; N, a nucleoprotein found in the virus core; NS, a phosphorylated protein also found in the virus core. Since the virus is negative stranded, it is generally accepted that the initial step in the replica-

tion of the virus is the production of complementary messenger RNAs by the virion-associated transcriptase, L, etc., using the negative RNA as a template. The mRNA is then adenylated, capped, and serves as a messenger to make protein. The molecular weights of the mRNAs which code for the production of these proteins in cell-free systems are, respectively, L, $> 1.65 \times 10^6$; G, 0.7×10^6; M, 0.28×10^6; N, 0.55×10^6; and NS, 0.28×10^6 (Rose and Knipe, 1975). At a certain stage in the virus replication, the production of adenylated mRNA is switched to the production of full-length 42 S RNA. A double-stranded replicating form of RNA is formed, followed by the synthesis of full-length negative viral RNA. The assembly of the various components and escape from the cell membrane then follow.

4.1.2. Transcriptional Mapping of the Genes of VSV

Irradiation with ultraviolet light has been used by Ball and White (1976) and by Abraham and Banerjee (1976) to map the order of transcription of the genes of VSV. Since it is known that the transcription and/or translation of UV-irradiated RNA is blocked or at least severely hindered by the introduction of the principal UV lesions, cyclobutadipyrimidines or pyrimidine photohydrates (Gordon *et al.*, 1976; see also Section 1.2), the UV target sizes for the loss of mRNA activity can in principle be used to determine the order of arrangement of genomes in VSV and the mode of transcription. Several alternative modes of transcription are possible. First, one could postulate that each mRNA had an independent initiation site; thus the target size for the loss of formation of each mRNA would be proportional to the size of the particular RNA in question. A second possibility is that there is only one initiation site for the initiation of mRNA synthesis. In this case, the introduction of UV lesions will have a strongly polar effect on the transcription of subsequent genes. The target size of genes will progressively increase, and, except for the first gene, the target size of subsequent genes will be larger than the physical size of the mRNAs. A third possibility is that a combination of the above two processes is occurring. Abraham and Banerjee (1976) irradiated Triton-treated VSV particles with UV and then used the disrupted virions to produce mRNA molecules which were fractionated into the known polyadenylated mRNAs. The results are shown in Fig. 7. A compilation of the results is also given in Table 1 taken from this paper. In a similar series of investigations, Ball and White (1976) arrived at similar conclusions. These investigators in the 1976 paper studied the expression of

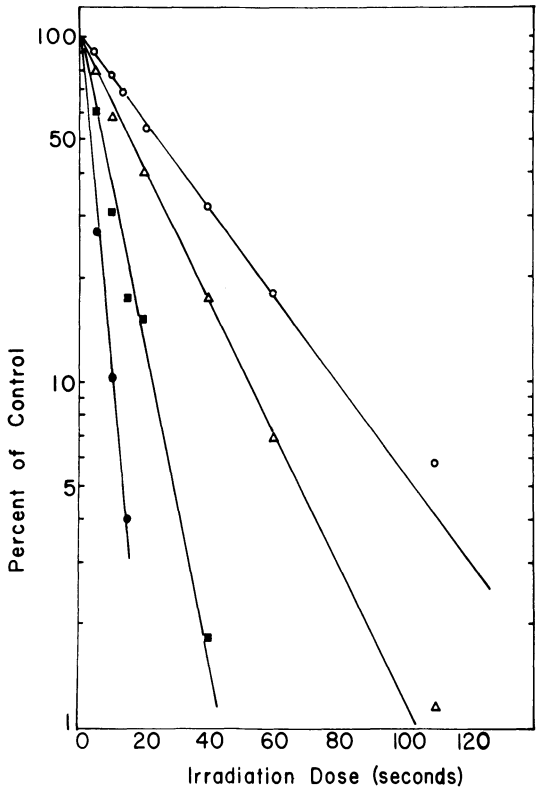


Fig. 7. Effect of UV irradiation of VSV on the synthesis of poly(rA)-containing mRNA species. ●, Synthesis of L message; ■, synthesis of G message; Δ, synthesis of the (NS + M) messages; O, synthesis of N message. Redrawn from Abraham and Banerjee (1976).

TABLE 1

Comparison of the Target Sizes and the Molecular Weights of the VSV mRNA Species[a]

Genes	Molecular weight of mRNA ($\times 10^{-6}$)	Observed target sizes[b] ($\times 10^{-6}$)	Estimated target sizes[c] ($\times 10^{-6}$)
N	0.55	0.55	0.55 (N)
NS	0.33	0.85	0.88 (N + NS)
M	0.33	0.85	0.88 (N + M)
G	0.70	2.06	1.91 (N + NS + M + G)
L	2.10	4.06	4.01 (N + NS + M + G + L)

[a] From Abraham and Banerjee (1976).
[b] The times of UV irradiation resulting in a 37% residual expression of each gene were N = 34 sec, (NS + M) = 22 sec, G = 9 sec, and L = 4–5 sec.
[c] Estimated target sizes in each case were derived by the inclusion of the molecular weights of 3′-adjacent genes as indicated by the parentheses.

the VSV genes in an *in vitro* coupled transcription–translation system or, as in the case of the 1977 manuscript, the synthesis of N, NS, M, G, and L proteins in mouse L cells. The results are shown in Table 2.

The results of both laboratories are beautifully in agreement and strongly support the mechanism of transcription of the viral genome starting at a single site. The gene order is determined to be N, NS, M, G, and L. Within the limits of the experimental procedure, there is a pronounced polar effect of UV radiation on the expression of the viral genes, with the target sizes of genes being the sum of the target sizes proceeding from a fixed origin. The target size for the loss of infectivity of the virus was assumed to be the molecular weight of the genome RNA. Nevertheless, the results seem to rule out the first and third hypotheses mentioned above and argue against any extensive excision and splicing during transcription of mRNA, such as has been repeatedly observed in a number of systems using DNA templates.

4.1.3. Inhibition of Host Cell Protein Synthesis by VSV

It has been observed that viruses frequently inhibit the synthesis of RNA and proteins in host cells. In 1965, Huang and Wagner showed

TABLE 2

Comparison of the Target Sizes for the Genes of VSV Measured *in Vivo* and *in Vitro*[a]

	Target size (daltons of RNA $\times 10^{-6}$) determined from			mRNA size (daltons of RNA $\times 10^{-6}$)	
Gene	Protein *in vivo*	Protein *in vitro*	mRNA *in vitro*	Individual[b]	Cumulative
N	0.6	0.55	0.48	0.55	0.55 (N)
NS	—[c]	0.83	0.96[d]	0.28	0.83 (N + NS)
M	1.2	1.12		0.28	1.11 (N + M)
G	2.1	1.76	1.72	0.7	1.81 (N + NS + M + G)
L	3.9	—[e]	3.9	2.1	3.91 (N + NS + M + G + L)

[a] From Ball (1977). The target size for the loss of viral infectivity was assumed to correspond to the molecular weight of VSV genome RNA, i.e., 3.82×10^{-6}. The other target sizes were calculated as being proportional to the corresponding UV sensitivity.

[b] Rose and Knipe (1975).

[c] The NS protein was obscured by an intense cellular polypeptide with which it comigrated.

[d] The mRNAs for NS and M proteins were not separated by methods used.

[e] No L protein synthesis was detected *in vitro*.

that VSV that had been UV treated so that no infectivity could be detected still inhibited RNA synthesis in Krebs 2 cells (Huang and Wagner, 1965). Similar results were obtained by Yaoi *et al.* (1970) and by Yaoi and Amano (1970). Later, Wertz and Youngner (1972) reported that the inhibition of host cell macromolecular synthesis was progressively inhibited by UV irradiation, and postulated an initial multiplicity-dependent, UV-insensitive inhibition as well as a progressive cycloheximide- and UV-sensitive inhibition. Marcus and Sekellick and coworkers have published a number of reports dealing with the inhibition of host cell protein synthesis (Marcus and Sekellick, 1974, 1975, 1976; Marvaldi *et al.*, 1977, 1978; Marcus *et al.*, 1977). These authors compared the sensitivity of plaque formation and cell killing activity of UV-irradiated VSV and found that the latter activity was about 5 times more resistant to UV inactivation (Marcus and Sekellick, 1975).

The results of these initial studies indicated that about one-fifth of the viral genome had to remain undamaged by UV and produce transcripts in order for a putative "cell-killing factor" to be formed. Subsequent studies using a series of temperature-sensitive mutants of VSV showed that both killing and inhibition of host cell protein synthesis required minimally a functional L protein and transcription of N and NS viral genes (Marvaldi *et al.*, 1977). This hypothesis was strengthened by the use of the so-called long, truncated defective interfering particle of VSV, DI-LT, which has a virion-associated L protein, and N, NS, M, and G genes, but no L gene. These particles, while incapable of replication, were effective as cell-killing particles (Marcus *et al.*, 1977). The results were in agreement with the hypothesis that cell killing by single particles of VSV requires transcription of genes N and NS by virus-associated transcriptase, L, and the translation of the resulting mRNAs into proteins. In the most recent paper in this series (Marvaldi *et al.*, 1978), a double-labeling technique was used to analyze the inhibition of cellular protein synthesis by UV-irradiated VSV. This method in general consisted of the preliminary labeling of a monolayer of L cells by [^{3}H]-amino acids, followed by infection with UV-irradiated VSV. Proteins synthesized subsequent to viral infection were labeled with [^{35}S]methionine. The proteins were then separated by polyacrylamide gel electrophoresis. The procedure was not only capable of detecting the inhibition of host cell protein synthesis, but also the UV-induced decrease of synthesis of the five viral proteins, L, G, NS, N, and M, although detection of NS protein following irradiation of the virus was obscured by technical difficulties. The relationship between the UV-induced loss of inhibition of host cell protein synthesis

and UV exposure is shown in Fig. 8. The synthesis of N protein was most resistant to UV irradiation of the whole virus, and was still detectable at UV doses corresponding to 10 and 20 lethal hits to viral infectivity at multiplicities of infection of 10 and 100, respectively. The great resistance of the synthesis of N to UV inactivation is in accord with the position of the N gene at the 3′-hydroxyl terminus of the negative-stranded viral genome. When the synthesis of viral protein was no longer discernible on gel electrophoresis, inhibition of host cell protein synthesis was no longer detectable. The hypothesis presented by the authors (Marvaldi *et al.*, 1978) postulates that cell killing and inhibition of protein synthesis involves the transcription and translation of the N and NS genes by functional viral transcriptase, L. The authors note that, at a survival level of 10^{-14} for VSV infectivity, the N and NS genes would survive to a level of 10^{-3}. Dubovi and Youngner (1976) estimate that there are from 5 to 50 transcriptionally active VSV particles per infectious virion. At a multiplicity of 100, there are thus $100 \times 10^{-3} \times (5 \text{ to } 50) = 0.5 \text{ to } 5$ surviving N genes and a somewhat smaller figure for the NS gene (see also Baxt and Bablanian, 1976). The inhibition of host cell protein synthesis and the extreme resistance of this inhibition to inactivation of UV light at high multiplicities can thus be explained by the presence in each affected cell of complete N and NS genes and a functional viral transcriptase (Marvaldi *et al.*, 1978).

4.1.4. Inhibition of Pseudorabies Virus Replication by VSV

Dubovi and Youngner (1976) have noted that VSV inhibited the replication of pseudorabies virus (PSR) in rabbit kidney cells. The

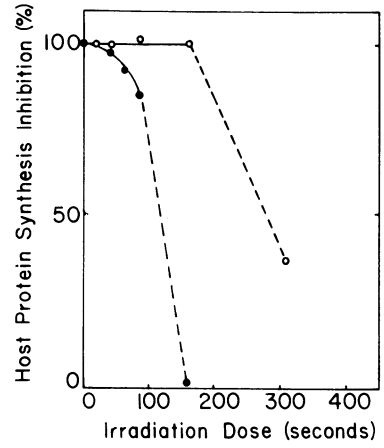


Fig. 8. Relationship between multiplicity of infection and inhibition of cellular protein synthesis by UV-irradiated VSV. Multiplicities of infection: ●, 10; ○, 100. The degrees of inhibition of protein synthesis were 52% and 60% for unirradiated virus at multiplicities of infection of 10 and 100, respectively. These values were normalized to 100%. The dashed lines indicate a high degree of uncertainty because of insufficient points on the curve, according to the authors. Redrawn from Marvaldi *et al.* (1978).

results obtained in these studies are quite similar to those obtained for the inhibition of host cell protein synthesis by VSV. One infectious VSV particle per cell will block the replication of PSR, while very high multiplicities of 800 to 1000 particles are required when noninfective UV-treated particles are used. The results can be interpreted as implying the necessity for replication of infecting VSV at low multiplicities and a direct inhibition of PSR replication by a virion component of VSV when high multiplicities of UV irradiated VSV were used. Alternatively, the same considerations expressed by Marvaldi *et al.* (1978) may be equally valid (see Section 3). In this case, the inhibition of PSR replication would be due to a functional L protein and intact copies of N and NS genomes.

4.1.5. Action Spectra for the Rescue of Temperature-Sensitive Mutants of VSV by UV-Irradiated Virions

A series of temperature-sensitive (*ts*) mutants of the Indiana serotype of VSV has been isolated and shown to form five complementation groups which appear to correspond to the five known viral proteins, M, G, L, N, and NS, respectively. When chicken embryo fibroblasts are infected with these temperature-sensitive mutants at a nonpermissive temperature, the mutants can be rescued by wild-type virus. The *ts* mutants can also be complemented by UV-irradiated wild-type or a *ts* mutant belonging to another complementation group. A determination of the UV action spectrum required to destroy the complementation activity gave some idea of the target of the UV light and the mechanism of the rescue process (Deutsch *et al.*, 1977; Deutsch, 1975, 1976).

The action spectrum for the rescue of a mutant temperature sensitive with respect to protein G was similar to the absorption spectrum of a protein; therefore, the rescue of the mutant probably is due to donation of intact glycoprotein G units by the rescuing virion. The inactivation of the rescue of this type of *ts* mutant is least sensitive to UV radiation than any other type of mutant. The authors note that the requirement of G protein for assembly into VSV can be met by glycoproteins of unrelated viruses, so that the configurational requirements imposed on the G protein are minimal.

The action spectrum for the loss of rescue of mutants temperature sensitive in protein L at all doses and all wavelengths indicated that protein L, the virion-associated transcriptase, is provided by the rescuing particle.

The action spectrum for the rescue of a mutant temperature-sensitive in protein NS indicated that the primary target in the complementing particle is the RNA. At very short wavelengths, 223 nm, the rescue ability of irradiated particles was very sensitive. This was taken to reflect either the destruction of the NS protein or the transcriptase, L, which is required to transcribe the gene coding for NS.

The rescue of mutants temperature-sensitive with respect to N was more complex. The dose–effect curves were indicative of two one-hit curves, indicating that two phenomena are involved in this complementation. The more sensitive component appears to be a nucleic acid, while the second, more radiation-resistant component appears to be due to a protein component. The shape of the action spectrum generally is in agreement with this interpretation.

Rescue of mutants temperature-sensitive with respect to protein M requires very high multiplicities and was not reported.

These results have led to the interesting conclusion that the rescue of *ts* mutants of VSV in some cases (notably those involving G, L and N) involved the reuse of viral structural proteins. In the case of mutants *ts* in NS and N, rescue appeared to involve the transcription of RNA. It should be noted that both N and NS proteins are closely associated with RNA, and reuse of these proteins may be difficult. Furthermore, the target sizes for the inactivation of the genes coding for N and NS are the smallest of the virion proteins. Thus these studies have shown very elegantly that the rescue of *ts* mutants at nonpermissive temperatures can be accomplished by genetic and nongenetic complementation.

4.1.6. Defective Interfering Particles

VSV, in common with many mammalian viruses, forms defective interfering particles. These particles are truncated in length and contain subgenomic amounts of nucleic acid. They are genetically inert and rely on complete virions for replication. Holland and coworkers (Holland *et al.*, 1976) have studied the UV inactivation of VSV-DI particles that were approximately one-third as large as infectious VSV. A dose of UV that caused an average of ten hits on an infectious VSV virion caused about one hit per VSV-DI. If one assumes a similar base composition and similar structure of nucleoprotein cores in VSV and VSV-DI particles, then an equal number of photochemical alterations would be expected per photon absorbed. The greater-than-predicted resistance of VSV-DI particles may be due to the fact that these particles can only replicate in the presence of infectious VSV particles, and some type of

complementation of the UV-inactivated DI particles may be intrinsic to the assay. These results are to be contrasted to those obtained with the midi variant of Qβ, MDV-1, which may be considered to be a DI particle of a bacterial RNA virus. The sensitivity of inactivation of the self-replication of MDV-1 is almost exactly what would be expected from its size, and the quantum efficiency for inactivation by an absorbed photon, ca. 2×10^{-3}, is similar to those of other single-stranded RNA viruses irradiated under similar conditions. The decreased sensitivity of VSV-DI may yield some interesting clues as to the mode of replication of these particles.

4.2. Picornaviruses

4.2.1. General

The picornaviruses are icosahedral particles 20–30 nm in diameter enclosing one single-stranded RNA of about 2.6×10^6 daltons. All virion proteins are present in multiple copies. The RNA is infectious and codes for a single large polypeptide that undergoes specific cleavage to produce specific viral proteins (Cooper, 1977; Spector and Baltimore, 1975; Rueckert, 1976). Several of the biological traits of the virus appear to reflect this mode of transcription of the virus RNA. Drake (1958) reported that multiplicity reactivation of UV-inactivated polio occurred at low frequency; however, this finding has been questioned recently (Cooper, 1977). Genetic recombination does occur to a limited extent in the case of polio (Cooper *et al.*, 1974), although the precise mechanism by which this occurs is not known. In addition to the processes mentioned by Cooper (1977), the recently discovered processing of RNA in eukaryotic cells should be considered. As will be noted below, most of the biological activities of polio are destroyed by UV irradiation by "one-hit" processes.

4.2.2. UV Inactivation of Infectivity

The initial studies of the UV inactivation of polio virus and its single-stranded RNA were performed by Norman (1960). These studies were supplemented in 1967 by experiments in which the UV inactivation of the infectious double-stranded RNA was also examined (Bishop *et al.*, 1967). The results obtained by Bishop *et al.* (1967) (Fig. 9) show that the double-stranded RNA is more resistant to UV inactivation than the single-stranded viral RNA, as would be expected (cf. Pearson

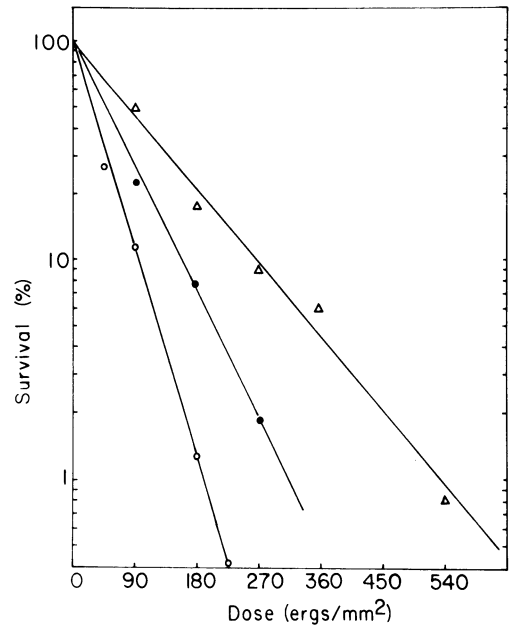


Fig. 9. Sensitivity of infectivity of poliovirus (○), single-stranded poliovirus RNA (●), and double-stranded poliovirus RNA (△) to UV radiation. Redrawn from Bishop *et al.* (1967).

and Johns, 1966; Závadova *et al.*, 1968); however, in both of the studies involving polio, the intact virion was found to be more sensitive to UV inactivation than even the single-stranded RNA. The reason for the apparent sensitization by the capsomer protein is not readily apparent. Two explanations may be proferred. There is a possible linkage of one of the viral proteins to the RNA in a reaction taking place with a high quantum yield made possible by the juxtaposition of a suitable amino acid residue to a nucleic acid base (see Gordon, 1976). Another possibility is the inclusion of some type of sensitizing compound within the intact virion (Schaffer, 1962). The putative sensitizer would be lost on preparation of viral RNA. This phenomenon remains an interesting subject for further investigation. The nature of the UV-induced lesion in the double-stranded polio RNA was not investigated, although the hyperchromicity on melting and quick cooling ruled out any possible interstrand cross-links at the UV dosage utilized. In the studies of Drake (1958) and Bishop *et al.* (1967), there was no detection of photoreactivation or any other type of host-mediated repair of UV-inactivated infectivity of virions or of single or double-stranded RNA.

4.2.3. Effects of Poliovirus Infection on Host Cell Metabolism

When animal cells are infected with polio virus and a number of other viruses, one of the first effects observed is the shut off of host pro-

tein synthesis. This is usually followed by depression of host cell RNA synthesis, and finally by cessation of host cell DNA synthesis. This process appears to provide a useful avenue for the exclusive synthesis of viral components. The initial infecting viral RNA must compete with host messenger RNA for ribosomes and other components of the protein synthesis complex; however, within a short time after infection, host polysomes disintegrate and host mRNA is no longer made (Spector and Baltimore, 1975). Subsequently, large viral polysomes make their appearance. This entire process takes place within 1–4 hr and is more rapid with a larger multiplicity of infection. The studies of Leibowitz and Penman (1971) have indicated that the shutoff of host protein synthesis depends on a viral protein that prevents the formation of polysomes from host mRNA.

Penman and Summers (1965) and Helentjaris and Ehrenfeld (1977) have both studied the UV-induced loss of the shutoff of host cell protein synthesis. A comparison of the doses necessary for the loss of infectivity and various activities is given in Table 3. Technical difficulties precluded the use of viral RNA in these studies. Both of these groups of investigators found that the loss of shutoff of host protein synthesis followed first-order kinetics, and this loss of shutoff appeared to coincide exactly with the loss of viral infectivity. These considerations appear to rule out a function for preformed viral proteins which are present in the infecting virion in more than one copy. The results are consistent with a hypothesis that the target is the viral RNA, and that one hit on this target destroys the ability to produce the active viral protein. The fact that polio virus is a polycistronic message complicates these studies. A hit anywhere in the RNA may have drastic polar

TABLE 3

UV Irradiation Survival of RNA and Protein Functions[a]

Target	D_{37} (ergs/mm^2)
Mengovirus	700
Poliovirus (infectivity)	306
Poliovirus (host protein shutoff)	306
Vesicular stomatitis virus	52.3
Rauscher leukemia virus	2,000
Carboxypeptidase A	300,000
Reverse transcriptase	23,000–31,000
Mengovirus hemagglutination activity	80,000

[a] From Helentjaris and Ehrenfeld (1977).

effects on translation, or an abnormal polypeptide may not be properly cleaved to form the protein that is responsible for host protein shutoff.

4.2.4. Effects of Extensive UV Irradiation of Polio Virus

Katagiri *et al.* (1967) have found that extensive irradiation of the Maloney strain of type 1 poliovirus led to a disruption of the virion. The conditions of irradiation were such that infectivity of the virions was rapidly lost and was not detectable after 2 min. The changes observed can be roughly divided into four stages. In the first stage (roughly 5 min of irradiation under their conditions), the infectivity of the particles was lost, but their sedimentation behavior in sucrose gradients and their appearance in the electron microscope was normal. The particles had N antigenicity. In the second stage (approximately 10 min), the banding of the particle was still normal but the encapsulated RNA had become RNase sensitive. A large part of the particles still possessed N antigenicity, but the N antigen was converted to H antigen by RNase treatment. It was suggested that the N antigenicity is determined by a specific interaction between capsomer proteins and RNA. The H antigen was found only in RNA-free viral capsids. In the third stage of irradiation (about 60 min), the sedimentation properties of the virus were changed, and the virions appeared to be empty capsids with adherent RNA, or simply empty capsids. The particles all had H antigenicity. In the fourth stage, after the extensive radiation time of about 120 min, the RNA was completely dissociated from the capsid shells, which were much lighter in sucrose gradients and only possessed H antigenicity.

4.2.5. Extensive UV Irradiation of Mengovirus

Another picornavirus whose photobiology has been studied is mengovirus. Miller and Plagemann (1974) have conducted a thorough study in the effects of extensive UV irradiation of this virus. The results of the irradiation of mengovirus are different from those obtained with polio in that no empty capsids are formed even on extensive irradiation.

The effects of 254-nm radiation on mengovirus can be conveniently divided into three phases. In the initial phase, the infectivity of the virus is rapidly destroyed in a fashion showing first-order kinetics. The dose for one hit was 700 ergs/mm^2, which is the same order of magnitude as for polio virus (Table 3). During this initial phase, 1.7 uracil dimers

were introduced into the viral RNA per lethal hit. The geometrical form and photoreversibility of the dimers were not determined. This figure is probably an underestimate because strong acid hydrolysis was used to isolate the dimers, and these conditions will result in some loss. In addition, under the conditions used, only the uracil moieties were highly labeled, so that cytosine-containing dimers would be underestimated even though they were probably deaminated to uracil dimers under the acidic hydrolysis conditions employed. No determinations were made of pyrimidine photohydrate formation. During this initial phase, there was no apparent change in the structural integrity of the virus.

With continued irradiation, the second phase was reached, and a gradual modification of the viral proteins could be detected. When leucine-labeled virus was irradiated for about 10 min and the viral proteins were analyzed by polyacrylamide electrophoresis, a small heterogeneous protein as well as a large protein were detected. Neither of these proteins was detected in unirradiated virus, and they probably represent heterogeneous breakdown and covalently linked polymers, respectively, of normal viral proteins. At about the same time, the hemagglutination activity gradually decreased, with a dose per hit of 84,000 ergs/mm^2. At this phase, the virion particles appeared to be normal in the electron microscope and sedimented normally in a sucrose gradient; nevertheless, subtle changes in the capsid were taking place as the RNA gradually became sensitive to RNase and could be extracted by treatment with 0.02% sodium dodecylsulfate. At this phase, the irradiated virions were also unstable when subjected to density gradient centrifugation in CsCl and formed some free protein and a ribonucleoprotein particle with a density of 1.39 g/ml, compared with 1.32 gm/ml for the native virions.

With further irradiation, 20–40 min, the third phase, involving viral breakdown, was entered. Sucrose gradient centrifugation indicated the presence of free 14 S protein particles and 80 S ribonucleoprotein particles, which contained all the viral RNA and 30% of the viral protein. Empty shells were never seen in the electron microscope, in contrast to the behavior of polio. The RNA extracted from virions at this stage had changed properties which appeared to reflect the covalent linkage of about 1.5% of the capsid protein (9×10^4 daltons) to the RNA. The sedimentation coefficient of the RNA increased from 34 S to 41 S. Relative to the untreated RNA, formaldehyde had a small effect on the sedimentation characteristics of RNA obtained from irradiated virus. It appears that the small amount of bound protein

introduced covalent cross-links into the RNA molecule so that the latter became more compact and less responsive to denaturants. These changes are reminiscent of the ultraviolet-induced cross-linking of the single-stranded circular DNAs of ϕX174 and M13 bacteriophages, although, in these instances, the role of protein was questionable (Francke and Ray, 1972; Gordon, 1976).

4.2.6. Inactivation and Repair of Encephalomyocarditis Virus

The double-stranded replicative form of encephalomyocarditis virus was first detected and shown to be infectious by Montagnier and Sanders (1963). The inactivation by UV of the virus, the viral RNA, and the double-stranded replicative form were studied by Závadova *et al.* (1968). The inactivation kinetics of the virion and the single-stranded viral RNA were first order, with 280 and 240 ergs/mm^2, respectively, required per lethal hit. The initial portion of the inactivation curve for the double-stranded RNA showed a small, concave portion, followed by a slower linear portion. The slower portion showed a dose of 1280 ergs/mm^2 per lethal hit, so, in common with polio virus, the double-stranded RNA was more resistant to ultraviolet light than the single-stranded virion RNA. The authors reported that the inactivation curves for the double-stranded RNA were difficult to reproduce. This was attributed to a variable extent of host cell reactivation in different cultures of indicator cells. When the double-stranded RNA was plated in the presence of caffeine, the loss of infectivity was distinctly more rapid. Caffeine had no effect on survival of the intact virion nor on the single-stranded RNA. A similar experiment was performed using normal human lung and xeroderma pigmentosum, XP-4, fibroblasts as indicator cells (see Fig. 10). These phenomena indicate some type of host cell repair is occurring in normal cells (Závadova, 1971). These reports have not yet been confirmed in other laboratories. In view of the importance of host cell repair of double-stranded RNA, confirmation and further investigation of this process is most important.

Chumakov and Agol (1976) prepared a series of heteroduplex molecules of the double-stranded RNA of encephalomyocarditis virus in which either the viral, plus strand, or the minus strand had been extensively inactivated by 254-nm light. Only duplex molecules which had intact plus strands were found to be infectious. These experiments unfortunately utilized doses of radiation that decreased the infectivity

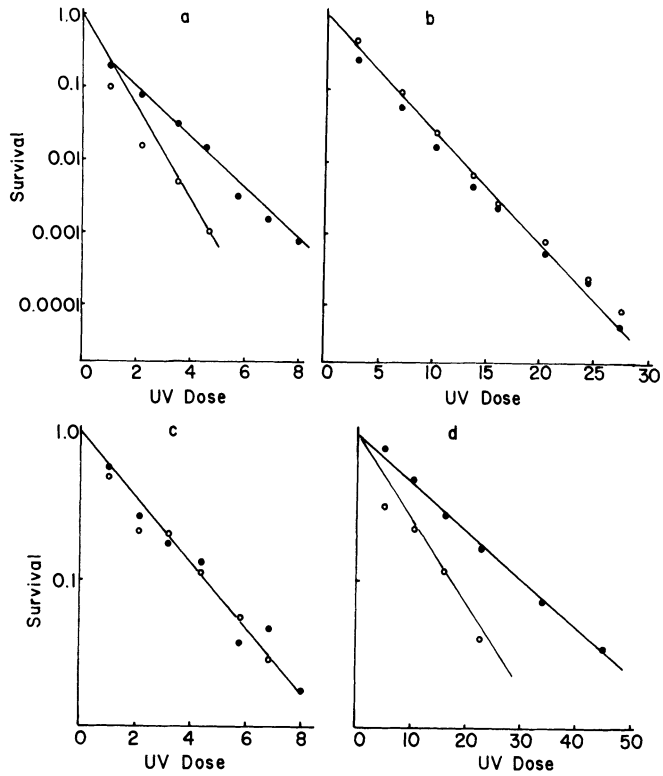


Fig. 10. Ultraviolet inactivation curves for (a) vaccinia virus, (b) encephalomyocarditis virus, (c) single-stranded encephalomyocarditis viral RNA, (d) doublestranded encephalomyocarditis viral RNA, inoculated onto normal (●) or xeroderma pigmentosum (○) fibroblasts. Ultraviolet exposure: 1 unit equals 10 J/m^2. Redrawn from Závadova (1971).

of the virion RNA by a factor of 10^4, or an average of eight hits per strand. It would be most interesting to repeat these experiments using plus or minus strands which had received an average of one to three hits to ascertain if there is any type of host repair mechanism as implied in the experiments of Závadova.

4.3. Retroviruses

4.3.1. General

The RNA tumor viruses have assumed an increasingly important role in recent years because of the ability of this type of virus to induce tumors in many animals. For details of the structure of these viruses, see Bader, 1975. Studies of the inactivation of retroviruses with

ultraviolet light have shed some light on the organization of the RNA of these particles as well as on the association of internal proteins with the RNA.

4.3.2. Avian Viruses

In early studies, Rubin and Temin (1959) and Levinson and Rubin (1966) found that the Bryan high-titer strain of *Rous sarcoma* virus (RSV) was about ten times more resistant to inactivation by 253.7-nm radiation than the L-Kam strain of Newcastle disease virus. These two viruses were chosen for comparison since the RNAs were roughly similar in size and both viruses were assayed on chicken cells. This resistence of RSV toward UV may be due to close association with protein as is the case for TMV; however, the molecular basis is unknown. Vogt (1973) proposed a polyploid model for the RNA of RSV, and a number of different types of investigations have suggested a diploid model for the genome in which two identical 30–40 S RNA molecules are held together at their 5′ termini to form a 60 S molecule (see Bister *et al.* (1977) for details). This proposed structure for the RNA genome should yield a multihit UV inactivation curve. Experiments done in several laboratories (Owada *et al.*, 1976; Bister *et al.*, 1977) have shown that, contrary to expectations, the UV inactivation curves obtained for a number of strains of RSV clearly indicated one-hit kinetics. Moreover, not only was the plaque-forming ability of the irradiated virus (infectivity) lost by one-hit kinetics, the focus-forming ability (transforming activity) also was lost by a process showing one-hit kinetics. The independent loss of these activities by a given virus particle was ruled out. Deletion mutants of RSV, which have slightly smaller RNA genomes, were inactivated at the same rates as nondefective RSV. Transformation and infectious center assays were carried out on chick embryo fibroblasts which expressed or did not express the chicken helper factor or which had been previously infected with RAV-1. In addition, Peking duck cells, which have few or no detectable viral sequences, were also used. The results of these assays were identical, indicating that there was no rescue or repair of UV damage by complementation or recombination with exogenous or endogenous viral sequences. Bister *et al.* (1977) also carried out a thorough analysis of the UV inhibition of total virus-specific DNA, full-length covalently closed circular viral DNA, virus-specific RNA, and particle production. The results showed that virus-specific DNA, RNA, and particle production are also inhibited, but these reactions are less sensitive to

UV than infectivity and transforming activity. At the present time, these results are difficult to explain. Bister *et al.* (1977) note that preparations of retroviruses have a preponderance of noninfectious particles. This preponderance may be a reflection of the high mutation rate of viruses with single-stranded RNA genomes and the known lack of fidelity of viral reverse transcriptase (Domingo *et al.*, 1978; Battula and Loeb, 1974). It may be that the single-hit inactivation kinetics are due simply to the fact that most infectious viral particles contain only one functional genome. Both groups (Bister *et al.*, 1977; Owada *et al.*, 1976) postulate that the simultaneous loss of infectivity and transforming activity is due to an inactivation event that blocks viral functions at an early phase even though the virus can subsequently produce biologically inert virus-specific RNA, DNA and virus-like particles.

The molecular basis of the above phenomenon is not understood, but some progress is being made. Owada *et al.* (1976) found that, following irradiation, the two RNA molecules comprising the diploid genome were linked by a pronase-sensitive bond. The work of Sen and Todaro (1977) indicates that a phosphoprotein, P19, which specifically binds *in vitro* to avian retroviral RNA, is probably the cross-linking protein. Inactivation of the viral reverse transcriptase probably accounts for a fraction of the UV inactivation of the virus (Owada *et al.*, 1976).

4.3.3. Murine Viruses

There have been several investigations of murine sarcoma and murine leukemia viruses (Kelloff *et al.*, 1970; Yoshikura, 1971; Nomura *et al.*, 1972). The results obtained with the murine viruses parallel the results obtained with the avian retroviruses. No evidence was obtained for the dissociation of transformation from replication on the part of sarcoma viruses, nor was there any dissociation of replication from ability of leukemia viruses to rescue defective sarcoma viruses. Thus, in the case of these RNA animal viruses, there also was no evidence of complementation or recombination between leukemia and sarcoma viruses. The results are in contrast to the behavior of polyoma virus (a DNA tumor virus) reported by Basilico and Di Mayorca (1965) and Benjamin (1965), where UV-induced loss of oncogenicity and replication could be separated. The murine viruses showed much greater UV resistance than Newcastle disease virus; thus the murine retroviruses also are similar to the avian viruses in this respect.

The nature of the viral components which are inactivated by the UV radiation is indicated by two types of experiments. First, Yoshikura (1971) grew a murine sarcoma virus in the presence of 5-fluorouracil and found that this virus was more sensitive to UV inactivation. It was postulated that the viral RNA had been sensitized by the incorporation of 5-fluoruoracil as has been found for TMV (Lozeron and Gordon, 1964; Bećarević, *et al.*, 1963). Thus one of the viral components inactivated by the UV light is the RNA. Lovinger *et al.* (1975) have determined that the UV doses for survivals of 37% of the infectivity of Rauscher leukemia virus and 37% of viral reverse transcriptase activity were 2×10^3 ergs/mm^2 and $2.4\text{–}3.1 \times 10^4$ ergs/mm^2, respectively. Thus the second viral component altered by 254-nm radiation is the viral reverse transcriptase, and this inactivation is responsible for at least 5–10% of the loss of viral infectivity. The chemical nature of the UV-induced damage is unknown.

5. CONCLUSION

5.1. Photochemistry of RNA

The major photochemical lesions induced in isolated viral RNA by ultraviolet radiation are pyrimidine hydrates and cyclobutadipyrimidines. Under the usual conditions of irradiation, other photoproducts (e.g., altered purines, pyrimidine–pyrimidine adducts) occur only at low rates, if at all.

The quantum efficiencies for the production of hydrates and cyclobutadipyrimidines (and "minor" photoproducts) depend on the environment of the RNA. The distribution of products is affected by the solvent (ionic strength, isotopic composition) and the presence of certain solutes (e.g., ketones as triplet sensitizers). The quantum efficiencies may also be affected by the encapsulation of the RNA with virus proteins, so that the chemical effects of irradiating whole virus may differ from those of irradiating isolated RNA. Thus cyclobutadipyrimidines are found in irradiated PVX and mengovirus but not in irradiated TMV or R17, and the efficiency of formation of hydrates in TMV is 1–2% of that in isolated TMV-RNA.

There are a number of points regarding the photochemistry of UV-irradiated RNA viruses and viral RNAs that remain unexplained. Photoproducts have been analyzed in only a few virus classes; for others, there is no information. Photoproducts considered "minor" in irradiated RNA may be much more important in irradiated viruses. It

would be particularly interesting to analyze poliovirus, since the whole virus is more sensitive to UV than the isolated RNA. Protein–nucleic acid complexes are formed in TMV and mengovirus, but the natures of the cross-links remain to be determined. The distribution of photoproducts along irradiated RNA or virus is unknown; if the distribution is nonrandom, the locations of the photoproducts must be determined. Finally, the reason certain capsid proteins (TMV) reduce the efficiency of formation of various photoproducts requires further study; speculations on the reasons (see Section 2.1.3b) must be considered hypothetical at this time.

5.2. Photochemistry of Capsid Proteins

UV has been shown to promote the denaturation of capsid proteins of TMV, poliovirus, and mengovirus. Denaturation can be assayed by changes in the appearance of virus particles in the electron microscope, by the RNase sensitivity of RNA in virus particles, by changes in the antigenicity, or by proteolysis or aggregation of protein subunits. Loss of the reverse transcriptase activity of RSV and Rauscher leukemia virus has also been demonstrated. In general, the doses required for extensive denaturation of viral proteins are at least an order of magnitude higher than those needed for inactivation of infectivity.

Studies on the mechanism of photolysis of TMV and brome mosaic virus proteins using fluorescence techniques have recently appeared. Further work should be very fruitful.

5.3. Biology of UV-Induced Killing

The most commonly studied biological effect of UV on RNA viruses is a loss of infectivity, the ability of the virus or viral RNA to complete at least one reproductive cycle. It is known that both pyrimidine hydrates and cyclobutadipyrimidines can be lethal to infectivity. This has been shown by identifying conditions under which one or the other is the only major identified photoproduct. There are also conditions known in which at least a fraction of the hydrates or dimers formed are not lethal. It is not known whether other photoproducts are as lethal as hydrates and dimers.

The effect of UV on RNA viruses has been studied using the following biological assays: infectivity, translation *in vitro* (messenger

activity), replication *in vitro* (template activity), transformation, inhibition of host protein synthesis, rescue of defective mutant virus, and mutation. The most sensitive function is infectivity; inhibition of host protein synthesis is either as sensitive (poliovirus) or less sensitive (VSV) than infectivity. Template activity seems to be more sensitive than messenger activity. There seems to be no mutagenic effect of UV on RNA in the system investigated (TMV-RNA). The lack of mutagenesis might reflect the lack of an error-prone repair process that works on UV-damaged TMV-RNA like the "SOS" repair process works on UV-damaged bacterial DNA.

There are virtually no details available concerning the mechanism by which UV photoproducts inhibit messenger or template activity of RNA. Do hydrates and dimers represent complete or partial blocks to protein and RNA synthesis? Another major gap in our knowledge about the lethal effects of UV concerns possible distributional or positional effects. Are there silent regions in which photoproducts are nonlethal? Are clusters of photoproducts needed for inactivation? We hope that answers to the above questions will explain why more than one photoproduct is needed to produce one biological hit in some viruses (TMV-RNA, mengovirus) although not in others (R17).

5.4. Repair of RNA

Photoreactivaton of UV-irradiated RNA viruses and viral RNA molecules is operationally defined: an infectious solution requires more radiation for a given degree of inactivation if the inoculated host is illuminated than if it is not. By this definition, photoreactivation of UV-irradiated RNA viruses appears similar to photoreactivation of UV-irradiated DNA viruses or of UV-irradiated cells.

Photoreactivation of RNA viruses appears to be specific to plant hosts. It has been reported to occur with bacterial viruses, but there has been no confirmation in the literature.

Experiments on the chemical basis of RNA photoreactivation have linked cyclobutadipyrimidines with photoreactivation. In this respect, RNA photoreactivation is similar to DNA photoreactivation. Beyond this point, the chemical basis of RNA photoreactivation remains undefined. A demonstration of *in vitro* photoreactivation of UV-irradiated TMV-RNA suggests that the process involves the repair of a UV-induced photoproduct, but the critical demonstration of the loss of photoproduct from RNA that has been reactivated is lacking, and it

probably must wait until the *in vitro* repair system is further refined. Similarly, the enzymatic or nonenzymatic basis of RNA photoreactivation remains to be determined.

The physiological role of an RNA repair system in plants remains a mystery. So far, all experiments have failed to show a repair of plant cell RNA or a repair of UV-induced damage to plant cells attributable to the RNA repair system. The failure may yet be a question of technique: recently, Jäckle and Kalthoff (1978) reported photorepair of UV damage of insect egg RNA.

There have been two reports suggesting dark repair of UV-irradiated RNA virus or viral RNA. One involves TNV and *Chenopodium amaranticolor*; the other concerns the double-stranded replicative form of encephalomyocarditis virus and human fibroblasts. These observations deserve confirmation and further investigation in view of the potential of dark repair processes to contribute both to genetic integrity and to genetic instability of viruses.

5.5. Uses of UV Radiation in RNA Virology

UV radiation is a convenient tool in the study of RNA viruses, in part because the equipment needed is very simple. Many experiments require only a low-pressure mercury vapor lamp and a sensor for determining the fluence rate. Jagger (1967) has written a handy introduction to the techniques.

In principle, action spectra can provide evidence for the identity of a functional viral component. One makes the assumptions that the components of a virus absorb light independently, that loss of function is due to light absorption and photolysis by the functional component, and quantum efficiency is constant over the effective spectral range. If these assumptions hold, the action spectrum should be proportional to the absorption spectrum of the functional component. Spectral peaks for the two main components of RNA viruses, RNA (269 nm) and protein (280 nm), should be easily distinguishable. A recent use of this technique has been the study of rescue of different temperature-sensitive mutants of VSV (see Section 4.1.5). Unfortunately, the above assumptions are not valid for all viruses. Interactions between the viral components, including sensitizing energy transfer and shielding, can confuse results (compare Figs. 3 and 6).

Another use of UV is to study the conformation of viral particles. One may test for interaction between RNA and protein by comparing the action spectra for inactivation of infectivity of virus and viral RNA

(see Figs. 2 and 3), or, better, by comparing chemically the RNA photoproducts induced in virus and viral RNA. One can demonstrate juxtaposition of protein and RNA or of two RNA components by demonstrating that UV forms cross-links between them [see Sections 2.1.3c (TMV), 2.3.4 (raspberry ringspot virus), and 4.2.5 (mengovirus)]. Conceivably, one might demonstrate juxtaposition of areas within a single type of RNA that is folded within a capsid by irradiating to form dimers, then observing the extracted RNA molecules in the electron microscope and measuring the distribution and positions of loops. A related technique, involving near UV in conjunction with psoralen sensitizers, may be used to locate double-stranded helical loops in single-stranded RNA. The technique was developed with the single-stranded DNA phage fd (Shen *et al.*, 1979), but psoralen sensitizers have been shown to inactivate RNA viruses (Nakashima and Shatkin, 1978; Hearst and Thiry, 1977), and they probably show the same affinity for double-stranded regions in RNA as in DNA (Isaacs *et al.*, 1977).

UV may also be used in the structural analysis of viral genes. One can determine the number of initiation sites involved in the transcription of several genes by comparing the cross-sections for inactivation of each gene (see Section 4.1.2). If a single initiation site is involved, as was found for VSV, it is possible to determine the order of transcription of the genes.

It is likely that other uses for UV radiation in virology will appear as more questions concerning the photobiology of RNA viruses are identified and answered.

6. REFERENCES

Abraham, G., and Banerjee, A. K., 1976, Sequential transcription of the genes of vesicular stomatitis virus, *Proc. Natl. Acad. Sci. USA* **73**:1504.

Adman, E., Gordon, M. P., and Jensen, L. H., 1968, Structure of the *cis-syn*-photodimer of uracil: A puckered cyclobutane, *Chem. Commun.* **1968**:1019.

Alper, T., Haig, D. A., and Clarke, M. C., 1966, The exceptionally small size of the scrapie agent, *Biochem. Biophys. Res. Commun.* **22**:278.

Augenstein, L., and Riley, P., 1964, The inactivation of enzymes by ultraviolet light. IV. The nature and involvement of cystine disruption, *Photochem. Photobiol.* **3**:353.

Bader, J. P., 1975, Reproduction of RNA tumor viruses, in: *Comprehensive Virology*, Vol. 4 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 253, Plenum Press, New York.

Ball, L. A., 1977, Transcriptional mapping of vesicular stomatitis virus *in vivo*, *J. Virol.* **21**:411.

Ball, L. A., and White, C. N., 1976, Order of transcription of genes of vesicular stomatitis virus, *Proc. Natl. Acad. Sci. USA* **73**:442.

Basilico, C., and DiMayorca, G., 1965, Radiation target size of the lytic and the transforming ability of polyoma virus, *Proc. Natl. Acad. Sci. USA* **54**:125.

Battula, N., and Loeb, L. A., 1974, The infidelity of avian myoblastosis virus deoxyribonucleic acid polymerase in polynucleotide replication, *J. Biol. Chem.* **249**:4086.

Baugher, J. F., and Grossweiner, L. I., 1975, Ultraviolet inactivation of papain, *Photochem. Photobiol.* **22**:163.

Bawden, F. C., and Harrison, B. D., 1955, Studies on the multiplication of tobacco necrosis virus in inoculated leaves of French bean plants, *J. Gen. Microbiol.* **13**:494.

Bawden, F. C., and Kleczkowski, A., 1953, The behaviour of some plant viruses after exposure to ultraviolet radiation, *J. Gen. Microbiol.* **8**:145.

Bawden, F. C., and Kleczkowski, A., 1955, Studies of the ability of light to counteract the inactivating action of ultraviolet radiation on plant viruses, *J. Gen. Microbiol.* **13**:370.

Bawden, F. C., and Kleczkowski, A., 1959, Photoreactivation of nucleic acid from tobacco mosaic virus, *Nature* (*London*) **183**:503.

Bawden, F. C., and Kleczkowski, A., 1960, Some effects of ultraviolet radiation on the infection of *Nicotiana glutinosa* leaves by tobacco mosaic virus, *Virology* **10**:163.

Baxt, B., and Bablanian, R., 1976, Mechanism of vesicular stomatitis virus-induced cytopathic effects. I. Early morphologic changes induced by infectious and defective-interfering particles, *Virology* **72**:370.

Bećarević, A., Djordjević, B., and Šutić, D., 1963, Effect of ultra-violet light on tobacco mosaic virus containing 5-fluorouracil, *Nature* (*London*) **198**:612.

Benjamin, T. L., 1965, Relative target size for the inactivation of the transforming and reproductive abilities of polyoma virus, *Proc. Natl. Acad. Sci. USA* **54**:121.

Bishop, J. M., Quintrell, N., and Koch, G., 1967, Poliovirus double-stranded RNA: Inactivation by ultraviolet light, *J. Mol. Biol.* **24**:125.

Bister, K., Varmus, H., Starmezer, E., Hunter, E., and Vogt, P. K., 1977, Biological and biochemical studies on the inactivation of avian oncovirus by ultraviolet irradiation, *Virology* **77**:688.

Boedtker, H., 1960, Configurational properties of tobacco mosaic virus ribonucleic acid, *J. Mol. Biol.* **2**:171.

Breck, L. O., 1971, The photochemistry of potato virus X, its ribonucleic acid, and a hybrid virus assembled from tobacco mosaic virus protein and PVX-RNA, Ph.D. Thesis, University of Washington, Seattle.

Breck, L. O., and Gordon, M. P., 1970, Formation, characterization, and some photochemical properties of a hybrid plant virus, *Virology* **40**:397.

Bruening, G., Beachy, R. N., Scalla, R., and Zaitlin, M., 1976, *In vitro* and *in vivo* translation of the ribonucleic acids of a cowpea strain of tobacco mosaic virus, *Virology* **71**:498.

Carpenter, J. M., and Kleczkowski, A., 1969, The absence of photoreversible pyrimidine dimers in the RNA of ultraviolet-irradiated tobacco mosaic virus, *Virology* **39**:542.

Cerutti, P. A., Miller, N., Pleiss, M. G., Remsen, J. F., and Ramsey, W. J., 1969, Photohydration of uridine in the RNA of coliphage R17. I. Reduction assay for uridine photohydration, *Proc. Natl. Acad. Sci. USA* **64**:731.

Challand, B. D., and DeMayo, P., 1968, Solvent effects on the stereochemistry of a photocycloaddition, *Chem. Commun.* **1968**:982.

Charlier, M., and Heléne, C., 1972, Photochemical reactions of aromatic ketones with nucleic acids and their components. I. Purine and pyrimidine bases and nucleosides, *Photochem. Photobiol.* **15**:71.

Charlier, M., Heléne, C., and Carrier, W. L., 1972, Photochemical reactions of aromatic ketones with nucleic acids and their components. III. Chain breakage and thymine dimerization in benzophenone photosensitized DNA, *Photochem. Photobiol.* **15**:527.

Chen, J., Huang, C. W., Hinman, L., Gordon, M. P., and Deranleau, D. A., 1976, Photomonomerization of pyrimidine dimers by indoles and proteins, *J. Theor. Biol.* **62**:53.

Chessin, M., 1965, Photoreactivation of clover yellow mosaic virus, *Photochem. Photobiol.* **4**:709.

Chumakov, K. M., and Agol, V. I., 1976, A study on the infectivity of encephalomyocarditis virus double-stranded RNA with selectively inactivated strands, *Virology* **73**:528.

Cooper, P. D., 1977, Genetics of Picornaviruses, in: *Comprehensive Virology*, Vol. 9 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 133, Plenum Press, New York.

Cooper, P. D., Steiner-Pryor, A., Scotti, P. D., and Delong, D., 1974, On the nature of poliovirus genetic recombinants, *J. Gen. Virol.* **23**:41.

DeBoer, G., Pearson, M. L., and Johns, H. E., 1967, Ultraviolet photoproducts in ordered structures of poly U and their effects on secondary structure, *J. Mol. Biol.* **27**:131.

DeBoer, G., Klinghoffer, O., and Johns, H. E., 1970, Reversal mechanisms for the photohydrates of cytosine derivatives, *Biochim. Biophys. Acta* **213**:253.

Deutsch, V., 1975, Nongenetic complementation of group V temperature-sensitive mutants of vesicular stomatitis virus by UV-irradiated virus, *J. Virol.* **15**:798.

Deutsch, V., 1976, Parental G protein reincorporation by a vesicular stomatitis virus temperature-sensitive mutant of complementation group V at nonpermissive temperature, *Virology* **69**:607.

Deutsch, V., Muel, B., and Brun, G., 1977, Action spectra for the rescue of temperature-sensitive mutants of vesicular stomatitis virus by ultraviolet-irradiated virions at nonpermissive temperature, *Virology* **77**:294.

Diener, T. O., and Hadidi, A., 1977, Viroids, in: *Comprehensive Virology*, Vol. 11 (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 285, Plenum Press, New York.

Diener, T. O., Schneider, I. R., and Smith, D. R., 1974, Potato spindle tuber viroid. XI. A comparison of the ultraviolet light sensitivities of PSTV, tobacco ringspot virus, and its satellite, *Virology* **57**:577.

Diner, J., and Murphy, T. M., 1973, Ultraviolet irradiation: Does RNA photoreactivation protect the leaf? *Photochem. Photobiol.* **18**:429.

Domingo, E., Sabo, D., Taniguchi, T., and Weissmann, C., 1978, Nucleotide sequence heterogeneity of an RNA phage population, *Cell* **13**:735.

Drake, J. W., 1958, Interference and multiplicity reactivation in polioviruses, *Virology* **6**:244.

Dubovi, E. J., and Youngner, J. S., 1976, Inhibition of pseudorabies virus replication by vesicular stomatitis virus. I. Activity of infectious and inactivated B particles, *J. Virol.* **18**:526.

Eggers, H. J., Ikegami, N., and Tamm, I., 1965, The development of ultraviolet-irradiation resistance by poliovirus infective centers and its inhibition by guanidine, *Virology* **25**:475.

Ehrenfeld, E., and Hunt, T., 1971, Double-stranded poliovirus RNA inhibits initiation of protein synthesis by reticulocyte lysates, *Proc. Natl. Acad. Sci. USA* **68**:1075.

Eoyang, L., and August, J. T., 1974, Reproduction of RNA bacteriophages, in: *Comparative Virology*, Vol. 2 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 1, Plenum Press, New York.

Evans, N. A., and McLaren, A. D., 1968, Photochemistry of tobacco mosaic virus RNA, *Photochem. Photobiol.* **7**:661.

Evans, N. A., and McLaren, A. D., 1969*a*, Quantum yields for the inactivation of tobacco mosaic virus ribonucleic acid by ultraviolet radiation (254 nm), *J. Gen. Virol.* **4**:55.

Evans, N. A., and McLaren, A. D., 1969*b*, A kinetic isotope effect in the photoreactivation of UV-inactivated tobacco mosaic virus, *Photochem. Photobiol.* **9**:243.

Fiers, W., 1979, Structure and function of RNA bacteriophages, in: *Comprehensive Virology*, Vol. 13 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 69, Plenum Press, New York.

Fischbach, F. A., and Anderegg, V. W., 1976, Comparisons of the low resolution structure of several small RNA-containing viruses, *Biochim. Biophys. Acta* **432**:404.

Francke, B., and Ray, D. S., 1972, Ultraviolet induced cross-links in the deoxyribonucleic acid of single-stranded deoxyribonucleic acid viruses as a probe of deoxyribonucleic acid packaging, *J. Virol.* **9**:1027.

Goddard, J., Streeter, D., Weber, C., and Gordon, M. P., 1966, Studies on the inactivation of tobacco mosaic virus by ultraviolet light. *Photochem. Photobiol.* **5**:213.

Gordon, M. P., 1976, Role of protein in the inactivation of viruses by ultraviolet radiation, in: *Aging, Carcinogenesis and Radiation Biology* (Smith, K. C., ed.), p. 105, Plenum Press, New York.

Gordon, M. P., Huang, C. W., Hurter, J., and Murphy, T., 1971, Studies on the photoreactivation of ribonucleic acids, *Fed. Am. Soc. Exp. Biol. Fed. Proc.* **30**:1154 (Abs).

Gordon, M. P., Huang, C. W., and Hurter, J., 1976, Photochemistry and photobiology of ribonucleic acids, ribonucleoproteins and RNA viruses, in: *Photochemistry and Photobiology of Nucleic Acids*, Vol. 2 (S. Y. Wang, ed.), p. 265, Academic Press, New York.

Govier, D. A., and Kleczkowski, A., 1970, Inactivation by ultraviolet radiation at different wavelengths of the RNA isolated from potato virus X. *Photochem. Photobiol.* **12**:345.

Green, A. E. S., Sawada, T., and Shettle, E. P., 1974, The middle ultraviolet reaching the ground, *Photochem. Photobiol.* **19**:251.

Grossweiner, L. I., 1976, Photochemical inactivation of enzymes, *Curr. Top. Radiat. Res. Q.* **11**:141.

Grossweiner, L. I., and Usui, Y., 1971, Flash photolysis and inactivation of aqueous lysozyme, *Photochem. Photobiol.* **13**:195.

Grouse, L., Murphy, T. M., Gordon, M. P., and Smuckler, E. A., 1970, The photobiology of a spherical phenotypic hybrid to tobacco mosaic virus, *Virology* **41**:385.

Harm, W., Harm, H., and Rupert, C. S., 1968, Analysis of photoenzymatic repair of UV lesions in DNA by single light flashes. II. *In vivo* studies with *Escherichia coli* cells and bacteriophage, *Mutat. Res.* **6**:371.

Harrison, B. D., and Nixon, H. L., 1959, Some properties of infective preparations made by disrupting tobacco rattle virus with phenol, *J. Gen. Microbiol.* **21**:591.

Hayashi, T., Machida, H., Abe, T., and Kiho, Y., 1969, *In vivo* uncoating of tobacco mosaic virus irradiated with ultraviolet light, *Jpn. J. Microbiol.* **13**:386.

Hearst, J. E., and Thiry, L., 1977, The photoinactivation of an RNA animal virus, vesicular stomatitis virus, with the aid of newly synthesized psoralen derivatives, *Nucleic Acids Res.* **4**:1339.

Heléne, C., and Charlier, M., 1971, Photosensitized splitting of pyrimidine dimers by indole derivatives, *Biochem. Biophys. Res. Commun.* **43**:252.

Helentjaris, T., and Ehrenfeld, E., 1977, Inhibition of host cell protein synthesis by UV-inactivated poliovirus, *J. Virol.* **21**:259.

Herzog, M., Gerard, D., Hirth, L., and Laustriat, G., 1977, Natural fluorescence properties of brome mosaic virus protein, *Biochim. Biophys. Acta* **493**:167.

Hidalgo-Salvatierra, O., and McLaren, A. D., 1969, Photoreactivation action spectrum of tobacco mosaic virus-ribonucleic acid inactivated by UV radiation, *Photochem. Photobiol.* **9**:417.

Ho, P. P. K., and Washington, A. L., 1971, Evidence for a cellular ribonucleic acid synthesis inhibitor from poliovirus-infected HeLa cells, *Biochemistry* **10**:3646.

Hollaender, A., and Duggar, B. M., 1936, Irradiation of plant viruses and of microorganisms with monochromatic light. III. Resistance of the virus of typical tobacco mosaic and *Escherichia coli* to radiation from 3000 to 2250 Å, *Proc. Natl. Acad. Sci. USA* **22**:19.

Holland, J. J., 1964, Inhibition of host cell macromolecular synthesis by high multiplicities of poliovirus under conditions preventing virus synthesis, *J. Mol. Biol.* **8**:574.

Holland, J. J., Villarreal, L. P., and Breinal, M., 1976, Factors involved in the generation and replication of rhabdovirus defective T particles, *J. Virol.* **17**:805.

Huang, A. S., and Wagner, R. R., 1965, Inhibition of cellular RNA synthesis by nonreplicating vesicular stomatitis virus, *Proc. Natl. Acad. Sci. USA* **54**:1579.

Huang, C. W., and Gordon, M. P., 1972, Photoreactivation of tobacco mosaic virus and potato virus X ribonucleic acids inactivated by acetone-sensitized photoreaction, *Photochem. Photobiol.* **15**:493.

Huang, C. W., and Gordon, M. P., 1974, The formation of photoreversible cyclobutane-type pyrimidine dimers in ultraviolet-irradiated potato virus X, *Photochem. Photobiol.* **19**:269.

Hurter, J., Gordon, M. P., Kirwan, J. P., and McLaren, A. D., 1974, *In vitro* photoreactivation of ultraviolet-inactivated ribonuculeic acid from tobacco mosaic virus, *Photochem. Photobiol.* **19**:185.

Isaacs, S. T., Shen, C. J., Hearst, J. E., and Rapoport, H., 1977, Synthesis and characterization of new psoralen derivatives with superior photoreactivity with DNA and RNA, *Biochemistry* **16**:1058.

Jäckle, H., and Kalthoff, K., 1978, Photoreactivation of RNA in UV-irradiated insect eggs (*Smittia* sp., Chironomidae, Diptera) I. Photosensitized production and light-dependent disappearance of pyrimidine dimers, *Photochem. Photobiol.* **27**:309.

Jagger, J., 1967, *Introduction to Research in Ultraviolet Photobiology*, Prentice-Hall, Englewood Cliffs, N.J.

Jellinek, T., and Johns, R. B., 1970, The mechanism of photochemical addition of cysteine to uracil and formation of dihydrouracil, *Photochem. Photobiol.* **11**:349.

Jennings, B. H., Pastra-Landis, S., and Lerman, J. W., 1972, Photosensitized dimerization of uracil, *Photochem. Photobiol.* **15**:479.

Johns, H. E., Pearson, M., and Brown, I. H., 1966, Mathematical aspects of the ultraviolet photochemistry of poly U, *J. Mol. Biol.* **20**:231.

Kacian, D. L., Mills, D. R., Kramer, F. R. and Spiegelman, S., 1972, A replicating RNA molecule suitable for a detailed analysis of extracellular evolution and replication, *Proc. Natl. Acad. Sci. USA* **69**:3038.

Kaluskar, A. G., and Grossweiner, L. I., 1974, Photochemical inactivation of trypsin, *Photochem. Photobiol.* **20**:329.

Kaneko, M., Matsuyama, A., and Nagata, C., 1978, Tyrosine-sensitized photodimerization of thymine in aqueous solution, *Photochem. Photobiol.* **27**:359.

Kassanis, B., and Kleczkowski, A., 1965, Inactivation of a strain of tobacco necrosis virus and of the RNA isolated from it, by ultraviolet radiation of different wavelengths, *Photochem. Photobiol.* **4**:209.

Katagiri, S., Hinuma, Y., and Ishida, N., 1967, Biophysical properties of poliovirus particles irradiated with ultraviolet light, *Virology* **32**:337.

Kelloff, G., Aaronson, S. A., and Gilden, R. V., 1970, Inactivation of murine sarcoma and leukemia viruses by ultraviolet irradiation, *Virology* **42**:1133.

Khattak, M. N., and Wang, S. Y., 1972, The photochemical mechanism of pyrimidine cyclobutyl dimerization, *Tetrahedron* **28**:945.

Kleczkowski, A., 1954, Stability of chymotrypsin and tobacco mosaic virus decreased by ultraviolet radiation, *Biochem. J.* **56**:345.

Kleczkowski, A., 1960, Effects of nonionizing radiation on plant viruses, *Ann. N.Y. Acad. Sci.* **83**:661.

Kleczkowski, A., 1962, Destruction of the antigenicity *in vitro* of human serum albumin and of tobacco mosaic virus by ultraviolet radiation, *Photochem. Photobiol.* **1**:291.

Kleczkowski, A., 1968, Dark reactivation of ultraviolet-irradiated tobacco necrosis virus, *J. Gen. Virol.* **3**:19.

Kleczkowski, A., 1971, Photobiology of plant viruses, in: *Photophysiology*, Vol. 6 (A. C. Giese, ed.), pp. 179–208, Academic Press, New York.

Kleczkowski, A., and Govier, D. A., 1969, Action spectrum for inactivation of the infectivity of potato virus X by UV radiation, *Photochem. Photobiol.* **10**:53.

Kleczkowski, A., and McLaren, A. D., 1967, Inactivation of infectivity of RNA of tobacco mosaic virus during ultraviolet-irradiation of the whole virus at two wavelengths, *J. Gen. Virol.* **1**:441.

Kornhauser, A., 1976, UV induced DNA-protein cross-links *in vitro* and *in vivo*, *Photochem. Photobiol.* **23**:457.

Kust, S. V., Dobrov, E. N., and Tikchonenko, T. I., 1972, RNA structure in potato virus X particles, *Mol. Biol.* **6**:42.

Lamola, A. A., 1969, Specific formation of thymine dimers in DNA, *Photochem. Photobiol.* **9**:291.

Latarjet, R., Muel, B., Haig, D. A., Clarke, M. C., and Alper, T., 1970, Inactivation of the scrapie agent by near monochromatic ultraviolet light, *Nature* (*London*) **227**:1341.

Lauffer, M. A., and Stevens, C. L., 1968, Structure of the tobacco mosaic virus particle; Polymerization of tobacco mosaic virus protein, *Adv. Virus Res.* **13**:1.

Laustriat, G., and Hasselmann, C., 1975, Photochemistry of proteins, *Photochem. Photobiol.* **22**:295.

Lee, T. Y., and Fugate, R. D., 1978, Excited states of biomolecules. *Photochem. Photobiol.* **27**:803.

Leibowitz, R., and Penman, S., 1971, Regulation of protein synthesis in HeLa cells. III. Inhibition during poliovirus infection, *J. Virol.* **8**:661.

Lennox, E. S., Luria, S. E., and Benzer, S., 1954, On the mechanism of photoreactivation of ultraviolet-inactivated bacteriophage, *Biochim. Biophys. Acta* **15**:471.

Levinson, W., and Rubin, H., 1966, Radiation studies of avian tumor viruses and of Newcastle disease virus, *Virology* **28**:533.

Levintow, L., and Bishop, J. M., 1970, Comparative aspects of poliovirus replication, *J. Cell. Physiol.* **76**:265.

Logan, D. M., and Whitmore, G. F., 1966, Dehydration of UV irradiated uridine and its derivatives, *Photochem. Photobiol.* **5**:143.

Lomont, A. J., and Fresco, J. R., 1972, Ultraviolet photochemistry as a probe of polynucleotide conformation, in: *Progress in Nucleic Acid Research and Molecular Biology*, Vol. 12 (J. N. Davidson and W. E. Cohn, eds.), p. 1, Academic Press, New York.

Lovinger, G. G., Ling, H. P., Gilden, R. V., and Hatanaka, M., 1975, Effect of UV light on RNA directed DNA polymerase activity of murine oncornaviruses, *J. Virol.* **15**:1273.

Lozeron, H., 1964, Ultraviolet sensitization and photoreactivation of tobacco mosaic virus ribonucleic acid containing 5-fluorouracil, Ph.D. thesis, University of Washington, Seattle.

Lozeron, H. A., and Gordon, M. P., 1964, Ultraviolet sensitization and photoreactivation of tobacco mosaic virus ribonucleic acid containing 5-fluorouracil, *Biochemistry* **3**:507.

Magne, A., Gerard, D., Hirth, L., and Laustriat, G., 1977, Fluorescence study of tobacco mosaic virus protein, *Biochim. Biophys. Acta* **495**:189.

Marcus, P. I., and Sekellick, M. J., 1974, Cell killing by viruses, I. Comparison of cell-killing, plaque-forming and defective-interfering particles of vesicular stomatitis virus, *Virology* **57**:321.

Marcus, P. I., and Sekellick, M. J., 1975, Cell killing by viruses. II. Cell killing by vesicular stomatitis virus: A requirement for virion-derived transcription, *Virology* **63**:176.

Marcus, P. I., and Sekellick, M. J., 1976, Cell killing by viruses. III. The interferon system and inhibition of cell killing by vesicular stomatitis virus, *Virology* **69**:378.

Marcus, P. I., Sekellick, M. J., Johnson, L. D., and Lazzarini, R. A., 1977, Cell killing by viruses. V. Transcribing defective interfering particles of vesicular stomatitis virus function as cell-killing particles, *Virology* **82**:242.

Marvaldi, J. L., Lucas-Lenard, J., Sekellick, M. J., and Marcus, P. I., 1977, Cell killing by viruses. IV. Cell killing and protein synthesis inhibition by vesicular stomatitis virus require the same gene functions, *Virology* **79**:267.

Marvaldi, J. L., Sekellick, M. J., Marcus, P. I., and Lucas-Lenard, J., 1978, Inhibition of mouse L cell protein synthesis by ultraviolet-irradiated vesicular stomatitis virus requires viral transcription, *Virology* **84**:127.

Mattern, M., Binder, R., and Cerutti, P. A., 1972, Cytidine photohydration in R17 RNA, *J. Mol. Biol.* **66**:201.

Mayo, M. A., Harrison, B. D., Murant, A. F., and Barker, H., 1973, Cross linking of RNA induced ultraviolet irradiation of particles of raspberry virus, *J. Gen. Virol.* **19**:155.

McCleary, L. O., and Gordon, M. P., 1973, Ultraviolet irradiation of potato virus X, its RNA, and a hybrid virus particle: Photoreactivation, kinetic isotope effects, and quantum yield of inactivation, *Photochem. Photobiol.* **18**:9.

McLaren, A. D., and Hidalgo-Salvatierra, O., 1964, Quantum yields for enzyme inactivation and the amino acid composition of proteins, *Photochem. Photobiol.* **3**:349.

McLaren, A. D., and Kleczkowski, A., 1967, Some gross changes in particles of tobacco mosaic virus caused by large doses of ultraviolet radiation, *J. Gen. Virol.* **1**:391.

McLaren, A. D., and Moring-Claesson, I., 1961, An action spectrum for the inactivation of infectious nucleic acid from tobacco mosaic virus by ultraviolet light, *Progr. Photobiol., Proc. Congr., 3rd, 1960*, p. 573.

McLaren, A. D., and Shugar, D., 1964, *Photochemistry and Photobiology of Proteins and Nucleic Acids*, Pergamon, Oxford.

McLaren, A. D., and Takahashi, W. N., 1959, Scattering of light by tobacco mosaic virus and X-protein from infected tobacco plants, *Biochim. Biophys. Acta* **32**:555.

McLaren, A. D., Kirwan, J., Hidalgo-Salvatierra, O., Owen, E. D., and Knight, C. A., 1970, Photoreactivation of ultraviolet irradiation-inactivated RNA from tobacco mosaic virus on white leaves of a variegated mutant of Xanthi tobacco, *J. Gen. Virol.* **7**:167.

McLean, G. D., and Crowley, N. C., 1969, Inactivation of lettuce necrotic yellows virus by ultraviolet irradiation, *Virology* **37**:209.

McMullen, D. W., Jaskunas, S. R., and Tinoco, I., Jr., 1967, Application of matrix rank analysis to the optical rotatory dispersion of TMV-RNA, *Biopolymers* **5**:589.

Merriam, V., and Gordon, M. P., 1965, Photoreactivation of tobacco mosaic virus ribonucleic acid following near ultraviolet irradiation, *Proc. Natl. Acad. Sci. USA* **54**:1261.

Merriam, V., and Gordon, M. P., 1967, Pyrimidine dimer formation in ultraviolet irradiated TMV-RNA, *Photochem. Photobiol.* **6**:309.

Metzger, K., 1963, Overlapping action of host cell reactivation and photoreactivation in bacteria, *Photochem. Photobiol.* **2**:435.

Miller, N., and Cerutti, P. A., 1968, Structure of the photohydration products of cytidine and uridine, *Proc. Natl. Acad. Sci. USA* **59**:34.

Miller, R. L., and Plagemann, P. G. W., 1974, Effect of ultraviolet light on mengovirus formation of uracil dimers, instability and degradation of capsid and covalent linkage of protein to viral RNA, *J. Virol.* **13**:729.

Mills, D. R., Kramer, F. R., and Spiegelman, S., 1973, Complete nucleotide sequence of a replicating RNA molecule, *Science* **180**:916.

Montagnier, L., and Sanders, F. K., 1963, Replicative form of encephalomyocarditis virus ribonucleic acid, *Nature (London)* **199**:664.

Morrison, H., Curtis, H., and McDowell, T., 1966, Solvent effects on the photodimerization of coumarin, *J. Am. Chem. Soc.* **88**:5415.

Murphy, T. M., 1973, Inactivation of TMV-RNA by ultra-violet radiation in sunlight, *Int. J. Radiat. Biol.* **5**:519.

Murphy, T. M., 1975*a*, Inactivation of tobacco mosaic virus ribonucleic acid by near- and middle- ultraviolet light: Sensitization of sulfanilamide and chlortetracycline, *Biochem. Biophys. Res. Commun.* **65**:1108.

Murphy, T. M., 1975*b*, Nucleic acids: Interaction with solar UV radiation, *Curr. Top. Radiat. Res.* **10**:199.

Murphy, T. M., and Gordon, M. P., 1971*a*, Light-mediated regulation of TMV-RNA photoreactivation, *Photochem. Photobiol.* **13**:45.

Murphy, T. M., and Gordon, M. P., 1971*b*, Ultraviolet irradiation of tobacco leaves: Inhibition of tobacco mosaic virus-ribonucleic acid photoreactivation, *Photochem. Photobiol.* **14**:721.

Murphy, T. M., Kuhn, D. N., and Murphy, J. B., 1973, Ultraviolet irradiation of the components of the wheat embryo *in vitro* protein synthesizing system, *Biochemistry* **12**:1782.

Murphy, T. M., Wright, L. A., Jr., and Street, H. E., 1978, Lethal and mutagenic effects of UV light on cultured rose cells, *Abstracts, Fourth International Congress of Plant Tissue and Cell Culture*, Calgary, Canada, August 1978, p. 149.

Nakashima, K., and Shatkin, A. J., 1978, Photochemical cross-linking of reovirus genome RNA *in situ* and inactivation of viral transcriptase, *J. Biol. Chem.* **253**:8680.

Nomura, S., Bassin, R. H., Turner, W., Haapala, D. K., and Fischinger, P. J., 1972, Ultraviolet inactivation of Moloney leukemia virus: Relative target size required for virus replication and rescue of 'defective' murine sarcoma virus, *J. Gen. Virol.* **14**:213.

Norman, A., 1960, Ultraviolet inactivation of poliovirus ribonucleic acid, *Virology*, **10**:384.

Ono, J., Wilson, R. G., and Grossman, L., 1965, Effects of ultraviolet light on the template properties of polycytidylic acid, *J. Mol. Biol.* **11**:600.

Oster, G., and McLaren, A. D., 1950, The ultraviolet light and photosensitized inactivation of tobacco mosaic virus, *J. Gen. Physiol.* **33**:215.

Owada, M., Ihara, S., Toyoshima, K., Kozai, Y., and Sugino, Y., 1976, Ultraviolet inactivation of avian sarcoma viruses: Biological and biochemical analysis, *Virology* **69**:710.

Pearson, M., and Johns, H. E., 1966, Suppression of hydrate and dimer formation in ultraviolet-irradiated poly(U + A) relative to poly U, *J. Mol. Biol.* **20**:215.

Pearson, M., Whillans, D. W., LeBlanc, J. C., and Johns, H. E., 1966, Dependence on wavelength of photoproduct yields in ultraviolet-irradiated poly U, *J. Mol. Biol.* **20**:245.

Penman, S., and Summers, D., 1965, Effects on host cell metabolism following synchronous infection with poliovirus, *Virology* **27**:614.

Price, 1965, Inactivation of southern bean mosaic virus by ultraviolet light, *Virology* **25**:1.

Rahn, R. O., Landry, L. C., and Carrier, W. L., 1974, Formation of chain breaks and thymine dimers in DNA upon photosensitization at 313 nm with acetophenone, acetone, and benzophenone, *Photochem. Photobiol.* **19**:75.

Ramachandran, N., and Ghiron, C. A., 1979, Triplet-triplet energy transfer in trypsin: The role of the indole triplet in the ultraviolet-induced photolysis of β-trypsin, *Photochem. Photobiol.* **29**:67.

Rauth, A. M., 1965, The physical state of viral nucleic acid and the sensitivity of viruses to ultraviolet light, *Biophysical J.* **5**:257.

Remsen, J. F., and Cerutti, P. A., 1972, Ultraviolet inactivation and miscoding of irradiated R17-RNA *in vitro*, *Biochem. Biophys. Res. Commun.* **48**:430.

Remsen, J. F., Miller, N., and Cerutti, P. A., 1970, Photohydration of uridine in the RNA of coliphage R17. II. The relationship between ultraviolet inactivation and uridine photohydration, *Proc. Natl. Acad. Sci. USA* **65**:460.

Remsen, J. F., Mattern, M., Miller, N., and Cerutti, P. A., 1971, Photohydration of uridine in the ribonucleic acid of coliphage R17. Lethality of uridine photohydrates and nonlethality of cyclobutane type photodimers, *Biochemistry* **10**:524.

Rhoades, D. F., and Wang, S. Y., 1971, A new photoproduct of cytosine. Structure and mechanism studies, *J. Am. Chem. Soc.* **93**:3779.

Rose, J. K., and Knipe, D., 1975, Nucleotide sequence complexities, molecular weights, and poly(A) content of the vesicular stomatitis virus mRNA species, *J. Virol.* **15**:994.

Rubin, H., and Temin, H., 1959, A radiological study of cell-virus interaction in the Rous sarcoma, *Virology* **7**:75.

Rueckert, R. R., 1976, On the structure and morphogenesis of picornaviruses, in: *Comprehensive Virology*, Vol. 6 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 131, Plenum Press, New York.

Rushizky, G. W., Knight, C. A., and McLaren, A. D., 1960, A comparison of the ultraviolet light inactivation of the infectious ribonucleic acid preparations of tobacco mosaic virus with those of the native and reconstituted virus, *Virology* **12**:32.

Ryan, F. J., 1976, The effect of ultraviolet irradiation on the structure and template activity of MDV-1 RNA, Ph.D. thesis, University of Washington, Seattle.

Ryan, F. J., Huang, C. W., and Gordon, M. P., 1977, Ultraviolet inactivation of the midi variant of Qβ-RNA. Determination of quantum yield and lesions produced, *Photochem. Photobiol.* **26**:263.

Ryan, F. J., O'Hara, P. J. A., and Gordon, M. P., 1979, Ultraviolet inactivation of the midi variant of Qβ-RNA: Characterization of template activity, *Photochem. Photobiol.* **29**:289.

Schaffer, F. L., 1962, Binding of proflavine by and photoinactivation of poliovirus propagated in the presence of the dye, *Virology* **18**:412.

Schuster, H., 1964, Photochemie von Ribonucleinsäuren I. Die Photoprodukte von Uridylsäure und Cytidylsäure und ihr Nachweis in UV-bestrahlten Ribonucleinsäure, *Z. Naturforsch. Teil B* **19**:815.

Sehgal, O. P., 1973, Inactivation of southern bean mosaic virus and its ribonucleic acid by nitrous acid and ultraviolet light, *J. Gen. Virol.* **18**:1.

Sen, A., and Todaro, G. J., 1977, The genome-associated, specific RNA binding proteins of avian and mammalian type C viruses, *Cell* **10**:91.

Setlow, R. B., 1955, A relation between cystine content and ultraviolet sensitivity of proteins, *Biochim. Biophys. Acta* **16**:444.

Setlow, R. B., 1968, The photochemistry, photobiology, and repair of polynucleotides, *Prog. Nucleic Acid Res. Mol. Biol.* **8**:257.

Setlow, R. B., Carrier, W. L., and Bollum, F. J., 1965, Pyrimidine dimers in UV-irradiated poly dI:C, *Proc. Nat. Acad. Sci. USA* **53**:1111.

Shen, C. J., Ikoku, A., and Hearst, J. E., 1979, A specific DNA orientation in the filamentous bacteriophage fd as probed by psoralen crosslinking and electron microscopy, *J. Mol. Biol.* **127**:163.

Siegel, A., 1957, Ultra-violet irradiation of dry tobacco mosaic virus, *Nature* (*London*) **180**:1430.

Siegel, A., and Norman, A., 1958, Action spectra for two strains of tobacco mosaic virus, *Virology* **6**:725.

Siegel, A., and Wildman, S. G., 1954, Some natural relationships among strains of tobacco mosaic virus, *Phytopathology* **44**:277.

Siegel, A., and Wildman, S. G., 1956, The inactivation of infectious centers of tobacco mosaic virus by ultraviolet light, *Virology* **2**:69.

Siegel, A., Ginoza, W., and Wildman, S. G., 1957, The early events of infection with tobacco mosaic virus nucleic acid, *Virology* **3**:554.

Singer, B., 1971, Chemical modification of viral ribonucleic acid. IX. The effect of ultraviolet irradiation of TMV-RNA and other polynucleotides, *Virology* **45**:101.

Singer, B., and Fraenkel-Conrat, H., 1969, The role of conformation in chemical mutagenesis, *Progr. Nucleic Acid, Res. Mol. Biol.* **9**:1.

Singer, B., and Fraenkel-Conrat, H., 1970, The messenger and template activities of chemically modified polynucelotides, *Biochemistry* **9**:3694.

Singer, S. J., Bald, J. G., Wildman, S. G., and Owen, R. D., 1951, The detection and isolation of naturally occurring strains of tobacco mosaic virus by electrophoresis, *Science* **114**:463.

Small, G. D., and Gordon, M. P., 1967, Prevention of photoreactivation of tobacco mosaic virus-ribonucleic acid by reconstitution, *Photochem. Photobiol.* **6**:303.

Small, G. D., Tao, M., and Gordon, M. P., 1968, Pyrimidine hydrates and dimers in ultraviolet-irradiated tobacco mosaic virus ribonucleic acid, *J. Mol. Biol.* **38**:75.

Smith, K. C. (ed.), 1976*a*, *Aging, Carcinogenesis, and Radiation Biology: The Role of Nucleic Acid Addition Reactions*, Plenum Press, New York.

Smith, K. C., 1976*b*, The radiation-induced addition of proteins and other molecules to nucleic acids, in: *Photochemistry and Photobiology of Nucleic Acids*, Vol. 2 (S. Y. Wang, ed.), p. 187, Academic Press, New York.

Smith, K. C., and Alpin, R. T., 1966, A mixed photoproduct of uracil and cysteine (5-S-cysteine-6-hydrouracil). A possible model for the *in vivo* cross-linking of deoxyribonucleic acid and protein by ultraviolet light, *Biochemistry* **5**:2125.

Smith, K. C., and Meun, D. H. C., 1968, Kinetics of the photochemical addition of [^{35}S]cysteine to polynucleotides and nucleic acids, *Biochemistry* **7**:1033.

Smith, K. C., and O'Leary, M. E., 1967, Photoinduced DNA-protein cross-links and bacterial killing: A correlation at low temperatures, *Science* **155**:1024.

Spector, D. H., and Baltimore, D., 1975, The molecular biology of poliovirus, *Sci. Am.* **232**:25.

Streeter, D. G., and Gordon, M. P., 1967, Ultraviolet photoinactivation studies on hybrid viruses obtained by the cross-reconstitution of the protein and RNA components of U(1) and U(2) strains of TMV, *Photochem. Photobiol.* **6**:413.

Streeter, D. G., and Gordon, M. P., 1968, Studies of the role of the coat protein in the u.v. photoinactivation of U(1) and U(2) strains of TMV, *Photochem. Photobiol.* **8**:81.

Suck, D., Rayment, I., Johnson, J. E., and Rossman, M. G., 1978, The structure of southern bean mosaic virus at 5 Å resolution, *Virology* **85**:187.

Tao, M., 1967, Kinetic isotope studies on the inactivation of transforming deoxyribonucleic acid, tobacco mosaic virus, and its nucleic acid by ultraviolet light, Ph.D. Thesis, University of Washington, Seattle.

Tao, M., Gordon, M. P., and Nester, E. W., 1966, Kinetic isotope studies on the inactivation of transforming deoxyribonucleic acid, tobacco mosaic virus, and its nucleic acid by ultraviolet light, *Biochemistry* **5**:4146.

Tao, M., Small, G. D., and Gordon, M. P., 1969, Photochemical alterations in ribonucleic acid isolated from ultraviolet-irradiated tobacco mosaic virus, *Virology* **39**:534.

Tikchonenko, T. I., 1975, Structure of viral nucleic acids *in situ*, in: *Comprehensive Virology* (H. Fraenkel-Conrat and R. R. Wagner, eds.), pp. 1–117, Plenum Press, New York.

Varghese, A. J., 1972, Acetone-sensitized dimerization of cytosine derivatives, *Photochem. Photobiol.* **15**:113.

Varghese, A. J., 1973, Photochemistry of nucleic acids and their constituents, in: *Photophysiology*, Vol. 7 (A. C. Giese, ed.), pp. 207–274, Academic Press, New York.

Varghese, A. J., and Rupert, C. S., 1971, Ultraviolet irradiation of cytosine nucleosides in frozen solution produces cyclobutane-type dimeric products, *Photochem. Photobiol.* **13**:365.

Varghese, A. J., and Wang, S. T., 1968, Thymine–thymine adduct as a photoproduct of thymine, *Science* **160**:186.

Vogt, P. K., 1973, The genome of avian RNA tumor viruses: A discussion of four models, in: *Possible Episomes in Eukaryotes: The Proceedings of the Fourth Lepetit Colloquium*, (L. Silvestri, ed.), pp. 35–41, North Holland, Amsterdam.

Volkert, W. A., and Grossweiner, L. I., 1973, Flash photolysis of ribonuclease A, *Photochem. Photobiol.* **7**:81.

Wagner, R. R., 1975, Reproduction of rhabdoviruses, in: *Comprehensive Virology*, Vol. 4 (H. Fraenkel-Conrat and R. R. Wagner, eds.), p. 1, Plenum Press, New York.

Wang, S. Y., 1976, *Photochemistry and Photobiology of Nucleic Acids*, Academic Press, New York.

Wang, T. M., and McLaren, A. D., 1972, Conformational changes induced in tobacco mosaic virus nucleic acid by ultraviolet radiation, *Biophysik* **8**:237.

Wechter, W. J., and Smith, K. C., 1968, Nucleic acids. IX. The structure and chemistry of uridine photohydrate, *Biochemistry* **7**:4064.

Weissmann, C., 1974, The making of a phage, *FEBS Lett. Suppl.* **40**:S10.

Werbin, H., Hidalgo-Salvatierra, O., Seear, J., and McLaren, A. D., 1966, Comparative photoreactivation of ultraviolet light-inactivated tobacco mosaic virus ribonucleic acid on *Chenopodium*, pinto bean, and tobacco plants, *Virology* **28**:202.

Werbin, H., Valentine, R. C., and McLaren, A. D., 1967, Photobiology of RNA bacteriophages. I. Ultraviolet inactivation and photoreactivation studies, *Photochem. Photobiol.* **6**:205.

Werbin, H., Valentine, R. C., Hidalgo-Salvatierra, O., and McLaren, A. D., 1968, Photobiology of RNA bacteriophages. II. UV-irradiation of f2: Effects on extracellular stages of infection and on early replication, *Photochem. Photobiol.* **7**:253.

Wertz, G. W., and Youngner, J. S., 1972, Inhibition of protein synthesis in L cells infected with vesicular stomatitis virus, *J. Virol.* **9**:85.

Wilson, T. M. A., Perham, R. N., and Butler, P. J. G., 1978, Intermediates in the disassembly of tobacco mosaic virus at alkaline pH, *Virology* **89**:475.

Winkler, U., 1964, Uber die fehlende photo- und wirtszellreaktivierbarkeit des UV-inaktivierten RNS-phagen FR, *Photochem. Photobiol.* **3**:37.

Wright, L. A., Jr., and Murphy, T. M., 1975, Photoreactivation of nitrate reductase production in *Nicotiana tabacum* var. *Xanthi*, *Biochim. Biophys. Acta* **407**:338.

Wright, L. A., Jr., and Murphy, T. M., 1978, Ultraviolet radiation-stimulated efflux of 86-rubidium from cultured tobacco cells, *Plant Physiol.* **61**:434.

Yamada, Y., Shigeta, A., and Nozu, K., 1973, Ultraviolet effects on biological function of RNA phage MS2. *Biochim. Biophys. Acta* **299**:121.

Yaoi, Y., and Amano, M., 1970. Inhibitory effect of ultraviolet-inactivated vesicular stomatitis virus on initiation of DNA synthesis in cultured chick embryo cells, *J. Gen. Virol.* **9**:69.

Yaoi, Y., Mitsui, H., and Amano, M., 1970, Effect of UV-irradiated vesicular stomatitis virus on nucleic acid synthesis in chick embryo cells, *J. Gen. Virol.* **8**:165.

Yoshikura, H., 1971, Ultraviolet inactivation of murine leukemia and sarcoma viruses, *Int. J. Cancer* **7**:131.

Závadova, Z., 1971, Host-cell repair of vaccinia virus and of double stranded RNA of encephalomyocarditis virus, *Nature* (*London*) *New Biol.* **233**:123.

Závadova, Z., and Závada, J., 1968, Host-cell repair of ultraviolet-irradiated pseudorabies virus in chick embryo cells, *Acta Virol.* (*Prague*) **12**:514.

Závdova, Z., Gresland, L., and Rosenbergova, M., 1968, Inactivation of single- and double-stranded ribonucleic acid of encephalomyocarditis virus by ultraviolet light, *Acta Virol.* (*Prague*) **12**:515.

Zech, A. H., 1961, Cytochemische untersuchungen zer reproduktion des tabakmosaikvirus, *Z. Naturforsch. Teil B* **16**:520.

Zierenberg, B. E., Krämer, D. M., Geisert, M. G., and Kirste, R. G., 1971, Effects of sensitized and unsensitized longwave UV irradiation on the solution properties of DNA, *Photochem. Photobiol.* **14**:515.

Zimmerman, E. F., Huter, M., and Darnell, J. E., 1963, RNA synthesis in poliovirus-infected cells, *Virology* **19**:400.

Zinder, N. D., 1965, RNA phages, *Annu. Rev. Microbiol.* **19**:455.

CHAPTER 7

The Photobiology of the DNA Viruses λ, T4, and φX174

Rufus S. Day III

Nucleic Acids Section, Laboratory of Molecular Carcinogenesis
Carcinogenesis Intramural Program, Division of Cancer Cause and Prevention
National Cancer Institute, NIH, Bethesda, Maryland 20205

1. INTRODUCTION

1.1. Solar Ultraviolet

Ultraviolet (UV) light has been a component of the radiation emitted by the sun toward our planet for approximately 4.6×10^9 years, the age of our solar system (see, for example, Krogdahl, 1962). The spectrum of light now emitted by the sun closely approximates the continuous theoretical spectral distribution (as derived by Planck) calculated for a perfect absorber in thermal equilibrium at 5800°K (see Eisberg, 1961). The actual emission in the UV region is reduced with respect to the expected theoretical value because the solar photosphere, having a finite thickness and being partially composed of good UV absorbers, shows strong UV absorption (Stair, 1969). Furthermore, although the outer edge of the photosphere does contribute somewhat to the UV spectrum, this is precisely the region of minimum solar temperature (about 4300°K). And, because of the strong dependence on temperature of the energy output, the photospheric emission in the UV region is low. Out-

ward from the solar photosphere is the extremely hot chromosphere, followed by the solar corona of average temperature 1–2 $\times$ 10^{6}°K. Because these latter regions have extremely low gas densities when compared with that of the photosphere, their contribution to the total solar UV, although very strong, does not increase the UV emission to the level expected by Planck's derivation (Stair, 1969; Smith and Jacobs, 1973).

Some 2 billion years ago, when cyanobacteria presumably began to fluorish (Schopf, 1978), their photosynthetic conversion of carbon dioxide and water to carbohydrates resulted in an oxygen atmosphere. Absorbing strongly in the region 120–200 nm, oxygen was photochemically and efficiently converted to ozone, a good absorber of UV in the 200–280 nm region (Calvert and Pitts, 1966), the process contributing further to the near absence of solar radiation of wavelengths less than 300 nm at the earth's surface. This absence has presumably been of very positive value to the survival of the various kinds of organisms encountered on this planet. This is so because 200–300 nm UV is known to damage DNA, the genes of all growing organisms, and to cause mutation, cancer, and loss of self-reproductive capacity (see Hanawalt *et al.*, 1979). DNA has strong absorption in the 230–290 nm region, coupled with a probability (about 0.002) of undergoing damaging chemical changes when such a photon is absorbed.

Although little UV strikes the earth's surface, four kinds of evidence point to the conclusion that some photons having wavelengths in the DNA absorption region do reach the earth: (1) many organisms are equipped with a mechanism which, in the presence of visible light, reverses DNA damage known to be produced by 230–290 nm UV (photoreactivation; see Rupert and Harm, 1966, Rupert, 1975); (2) bacteria capable of repairing 230–290 nm UV-produced DNA damage (in the dark) are killed more slowly by sunlight than are their counterparts which are defective in such repair (Harm, 1969); (3) both sunlight and 230–290 nm UV produce in DNA a photoproduct called the pyrimidine dimer (Setlow and Carrier, 1966; Trosko, 1970); and (4) humans who have recessively inherited the syndrome called xeroderma pigmentosum are extraordinarily susceptible to skin cancer on areas of their skin exposed to sunlight and have, without known exception, a cellular defect in their ability to repair UV damage in their DNA (see, for example, Hanawalt *et al.*, 1979).

Thus the study of the biological effects of ultraviolet light can be expected to lead to a better understanding of the chemistry and biology of the life in the environment of our planet. It is the purpose of this

chapter to review the large role that studies of the effects of 254-nm UV irradiation of certain *E. coli* DNA bacteriophages have played in clarifying the mechanisms underlying both inactivation and reactivation of UV damaged biological entities. λ and T4 are double-stranded DNA phages, the former being much more greatly affected by the repair processes of its host than the latter. φX174, a single-stranded DNA phage (but infectious also in the double-stranded replicative form) affords examination of the effects of DNA strandedness on repair processes. The effects of UV irradiation on RNA were discussed in Chapter 6.

1.2. Laboratory UV Sources

1.2.1. Bulbs

In practice, one of the most convenient sources of UV is the GE or Sylvania 15T8 15 watt germicidal lamp, which fits the common 18-inch fluorescent bulb fixture. At a distance of 0.5 m, this bulb emits 254-nm UV at about 1–2 joules/m^2/sec. Both companies make UV bulbs in a range of sizes. Another source of bulbs for 254-nm UV irradiation is the Southern New England Ultraviolet Co. (Middletown, Connecticut), which also makes a 300-nm bulb. For a good discussion of UV sources and their handling, see Jagger, 1967.

A germicidal bulb usually will have a fairly constant output over a year's time if it is "broken in" piror to laboratory use by 100 hr continuous operation. Provision for the escape of the heated air surrounding an operating UV lamp will ensure constant temperature and thereby constant intensity during an experiment. Most of the blue light emitted by such germicidal bulbs can be eliminated, if desired, by means of a CS 7-54 (Glass number 9863) Corning Glass Works filter (Corning, New York).

About 85% of the emission from such bulbs is 254-nm UV. Although the penetration of 254-nm UV through tissue such as skin is low compared to that of visible or infrared radiation, laboratory conditions are easily arranged to ensure the uniform irradiation of suspensions of cells, bacteria, or other small biological entities. Of all biological macromolecules, nucleic acids have, on a weight basis, the greatest UV absorbance, 50 μg DNA/ml, giving an absorbance (OD) of about 1 at 260 nm, using a 1-cm path length, or about twentyfold more than proteins. Because the DNA of DNA bacteriophages appears to be the only active biochemical unit introduced into the internal bacterial

biochemical environment, the lethal or mutagenic effects of UV irradiation of such infectious units are likely to reflect the UV-produced changes in DNA. In addition, following the infection of a susceptible cell, UV-produced damage in the viral DNA may be repaired in one of several ways depending on (1) the structure of the infecting DNA in the virion (strandedness, degree of hydration), (2) the nature of the DNA photoproduct itself, and (3) the biochemistry of the infected (or host) cell (Rahn and Patrick, 1976).

1.2.2. Calibration of Sources

Three types of convenient laboratory UV calibration measurements are made: the measurement of the current output of a photovoltaic cell or phototube, the spectrophotometric measurement of a UV-produced chemical reaction (actinometry), or a measurement of survival of a standard biological organism such as phage T2. For a good review of these techniques the reader is referred to Jagger (1967).

1.2.2a. Photocurrent

A simple photovoltaic cell system similar to that described by Jagger (1961) can be constructed at low cost using a selenium photovoltaic cell (Type GQ, 25-mm diameter, open window, No 780 from Electrocell-GmbH, Königin-Luise-Strasse, 29, 1 Berlin 33, Germany) shielded with a 2- by 2-inch piece of Corning CS 7-54 filter (Corning, New York, Glass No. 9863) leading into a model 2124 Simpson Meter (0–50 DC microamps, Cat. No. 17590, Simpson Electric Co., Chicago). This meter may also be calibrated for the measurement of black light. The Latarjet meter, which may either be constructed (Latarjet *et al.*, 1953) or purchased already calibrated (Prof. R. Latarjet, Fondation Curie—Institut du Radium, 26, rue d'Ulm, Paris Cedex 05, France), is a sensitive and durable meter for use only with the germicidal UV. Alternatively, a calibrated germicidal UV meter may be purchased from Ultraviolet Products (San Gabriel, California), but this meter is less sensitive than either the Jagger or Latarjet meters.

1.2.2b. Photochemical

Two photochemical dosimetric methods for 254-nm UV are in current use. In both, a solution of a photosensitive chemical (dosimeter

solution) that will absorb all incident UV photons is illuminated for a given time. By spectrophotometric measurement, the concentration of the UV-generated photoproduct is determined. Knowing the number of absorbed photons required to produce one molecule of photoproduct and the surface area of the dosimeter solution exposed to UV, one can calculate the dose, i.e., the energy corresponding to the number of photons calculated to have been incident on the solution per unit area per second. Johns (1969) has described the malachite green leukocyanide method in detail. This is good for use in calibration of UV light from 220 to 313 nm. The concentration of a blue photoproduct is proportional to UV dose. The potassium ferrioxalate method of Hatchard and Parker (1956) as described by Jagger (1967) and made much more rapid by Day and Muel (1974) is also very good, and furthermore can be used for wavelengths up to and beyond 430 nm. Here, UV irradiation of acidic ferrioxalate results in the reduction of ferric to ferrous ion, to which is later added orthophenanthroline. The red ferrous ion–orthophenanthroline complex absorbs maximally at 510 nm.

Another dosimetric method for 300–400 nm wavelenths involving the irradiation of a benzene solution of 0.1 M benzophenone and 0.2 M benzyhydrol has also been described (Rosenthal and Bercovici, 1976). Benzophenone absorbs light between 300 and 400 nm, but neither the benzhydrol nor the photoproduct, benzpinacol (which is formed by the reaction of excited benzophenone with benzhydrol), has absorption in this region, so that the dose is proportional to the loss of benzophenone absorbance.

Rahn and Sellin (1979) found the quantum yield for the photoproduced hydrate of 1,3-dimethyluracil to be independent both of wavelength and concentration, and suggested its use as a chemical dosimeter over the range of 240–280 nm.

1.3. The Poisson Distribution

1.3.1. Single Hit or Exponential Inactivation

Latarjet and Wahl (1945) found the UV inactivation of phages C16 and S13 to be exponential; that is, each constant size increment of UV reduced the existing plaque-forming titer by the same multiplicative factor. Exponential inactivation of bacteriophages is usually, but not always, found. This is just the result to be expected if the Poisson dis-

tribution holds for the experimental problem. The Poisson distribution is

$$P(n) = \frac{x^n e^{-x}}{n!}$$

where $P(n)$ is the probability of the occurrence exactly n events if the average number of events is x. In the case of bacteriophage, the "events" are considered to be occurring in phage particles and, for the sake of more specific terminology, are called "lethal hits." The surviving fraction from the Poisson distribution is $P(0) = e^{-x}$, the probability of an infectious phage particle receiving exactly zero lethal hits. In plotting a survival curve, x is taken as a constant times the UV dose (kD), so that the natural logarithm of the surviving fraction, ln $P(0)$, is plotted vs. dose, D.

Thus, when the average number of lethal hits per phage particle (i.e., x or kD) equals 1, then the surviving fraction = $P(0) = e^{-1} = 0.368$. The dose at which the survival is 37%, then, is said to have introduced one lethal hit per infectious unit into the virus population, and is termed the D_{37}. The D_{37} is characteristic of a particular virus/host combination. Ideally, one would like to know what photoproduct (or photoproducts) is responsible for viral inactivation. If there is only one molecule of a certain photoproduct per virion at the D_{37} (where there is also one lethal hit per virion), then that photoproduct may be the "lethal hit" of interest.

1.3.2. Multitarget or Multihit Inactivation

In practice, straight line inactivation curves on ln $P(0)$ vs. linear dose plots are not always obtained. For example, curves showing resistant shoulders followed by a straight line indicative of greater sensitivity are often observed in the case of bacteriophage λ. The shoulder is describable mathematically as either a "multitarget" or "multihit" curve.

1.3.2a. Multitarget

If a biological entity is composed of r targets in such a way that requires each and every one of the targets to be inactivated in order that the entire entity be inactivated, then the survival of that entity as a function of dose is calculated as follows: The probability of survival of

one of the targets (assuming single hit inactivation of individual targets) is e^{-kD}, so the probability of its inactivation is $1 - e^{-kD}$. The probability of inactivating all r targets in an independent way is then $(1 - e^{-kD})^r$, and the surviving fraction is

$$P_{\text{multitarget}} = 1 - (1 - e^{-kD})^r$$

If it were not for phage–phage and phage–cell interactions, the multitarget situation would be expected to be applicable if cells were infected at a given multiplicity (greater than 1) or if phage developing intracellularly were irradiated.

1.3.2b. Multihit

The multihit situation arises when a given number of hits, say m, must be delivered to one target in order to inactivate the entity. In this case, the survival is written

$$P_{\text{multihit}} = P(0) + P(1) + P(2) + \cdots + P(m-1) = e^{-kD} \sum_{n=0}^{m-1} \frac{(kD)^n}{n!}$$

because the phage with 0, 1, 2, . . ., $m - 1$ hits are not killed. Thus, for a case in which two hits are required to inactivate an entity,

$$P_{2\,\text{hit}} = (1 + kD)e^{-kD}$$

A multihit situation could conceivably arise if the number of UV-produced DNA alterations exceeded the number of molecules of stoichiometrically acting, survival-limiting, cellular protein.

For more discussion of the applications of the Poisson distribution to survival curves, see Atwood and Norman (1949), Rupert and Harm (1966), Luria (1947), Luria and Dulbecco (1949), and Stahl (1959).

1.4. Photochemistry of DNA

Although the 254-nm photochemistry of DNA is certainly pertinent to the UV inactivation of bacteriophage, it is described only briefly here. The subject has been extensively reviewed recently (see Patrick and Rahn, 1976; Rahn and Patrick, 1976; Rahn, 1979) and is also covered in the preceding chapter dealing with RNA. Ultraviolet light produces pyrimidine dimers (a pyrimidine dimer consists of two

pyrimidines joined together by carbon–carbon bonds at both their 5 positions and their 6 positions to form a cyclobutane ring) in DNA (Beukers and Berends, 1960; Setlow and Carrier, 1966) at the rate of about 65 per 10^7 nucleotides per joule/meter^2 in *Escherischia coli* DNA (see, for example, Rupp and Howard-Flanders, 1968). At doses which are used to inactivate biological organisms, the rate of dimerization in various DNAs depends somewhat on the percentage of G + C in the DNA (Unrau *et al.*, 1973). At a given dose, more dimers are produced when irradiating AT-rich than GC-rich DNAs, but, between 35 and 70% G + C, this difference is not more than twofold. Dimers produced in DNAs are of various sorts: thymine:thymine, thymine:cytosine, cytosine:thymine, and cytosine:cytosine. At low doses (up to 200 J/m^2), the probability of a T-T neighboring pair being converted to a dimer is about twice that of a C-T or C-C pair. Beyond that dose, the probability of dimerization or C-T or C-C pairs diminishes because the probability of further dimerization becomes equal to that of the monomerization of the existing dimers (see Unrau *et al.*, 1973). The monomerization of thymine dimers by 254-nm UV occurs with only low probability because the absorbance at 254 nm of a thymine dimer is much less than that of C-T or C-C dimers.

Photoproducts other than dimers are known to be formed in DNA by UV. These include pyrimidine adducts (nondimer pyrimidine–pyrimidine structures) which may involve either or both DNA pyrimidines, and which occur at about one-tenth the frequency of dimers (Varghese and Wang, 1967; Varghese and Day, 1970; Patrick and Rahn, 1976). Other products include those of the 6-dihydro-5,6-dihydroxythymine type (which occur about one-thirtieth as frequently as dimers, (Hariharan and Cerutti, 1977), strand breaks, and cross-links (Patrick and Rahn, 1976).

The biological effects of dimers have been better documented than biological effects due to any other photoproduct. However, as we shall see, there is biological evidence implicating other photoproducts both in lethality and in induction of recombination.

1.5. UV-Sensitive Mutants of *E. coli*

1.5.1 The *uvr* Series of Mutants

Evelyn Witkin (1947) isolated the first *E. coli* mutant known to have altered sensitivity to ultraviolet light. It was a mutant of wild-type *E. coli* B, the latter now known to be UV sensitive with respect to wild-

type K12 strains (see Rupert and Harm, 1966). The mutant, *E. coli* B/r, had the UV sensitivity characteristic of *E. coli* K12 strains. Eleven years later, Ruth Hill (1958) isolated a UV-sensitive mutant, *E. coli* Bs-1, as one of 12 survivors of a population of 2.2×10^6 *E. coli* B treated with a single UV dose. The UV doses needed to reduce the survival of B/r, B, and Bs-1 to 37% are approximately 40 J/m^2, 8 J/m^2, and 0.1 J/m^2, respectively. Hill then went on to isolate a series of UV-sensitive mutants Bs-1 through Bs-12 (Hill and Simson, 1961; Hill and Feiner, 1963). However, the genetic constitution of *E. coli* B was not nearly so experimentally accessible as that of *E. coli* K12, so that genetic analysis of neither Witkin's nor Hill's mutants was carried out until later: Mattern *et al.* (1966) showed that *E. coli* B differed from *E. coli* Bs-1 by two mutations, *uvrB* and *exrA*. *E. coli* Bs-2 was found mutant only at *exr*. (Rorsch *et al.* (1962) reported the isolation of another extremely UV-sensitive mutant, *E. coli* B *syn*$^-$, which was subsequently introduced into *E. coli* C (Rorsch *et al.*, 1963). The mutant appeared to be due to a change in a single gene.)

Studying repair by *E. coli* K strains, Howard-Flanders and Theriot (1962) worked out a method to select *E. coli* mutants unable to repair UV-damaged T1 phage. Repair-proficient AB1157 *E. coli* were killed by restoring biological activity to infecting UV-damaged phage, enriching for mutants that were incapable of such repair. The mutant site of one such UV-sensitive isolate, AB1886, was mapped between arginine and arabinose (Howard-Flanders *et al.*, 1962) and was later called *uvrA*.

Using such mutant strains, Setlow and Carrier (1964) showed that, whereas on postirradiation incubation in complete medium, *E. coli* B/r and *E. coli* B could remove UV-produced thymine dimers from their DNA, *E. coli* Bs-1 of Hill could not.

In the same issue, Boyce and Howard-Flanders (1964), using the recently isolated *E. coli* K12 strain AB1886, showed very similar results. After being UV irradiated, AB1157 could excise thymine dimers from its DNA, but AB1886 could not. Later, using about 45 UV-sensitive mutants, Howard-Flanders *et al.* (1966) reported that at least three loci in *E. coli* K12, *uvrA*, *uvrB*, and *uvrC*, were responsible for controlling dimer excision.

Van de Putte *et al.* (1965) mapped six of 26 radiation-sensitive mutants isolated after *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine (MNNG) treatment of their parent strain KMBL49. The *dar1* and *dar6* mutant genes mapped at *uvrB* (no UV-resistant recombinants were obtained by the conjugation of *uvrB* with *dar1* or *dar6* strains), *dar3* near *uvrA*, *and*

dar4 and *dar5* at *uvrC*. *Dar2* mapped near isoleucine. The mutation in Harm's *E. coli* K12S *hcr*$^-$ (Harm, 1963*a*) was mapped at *uvrA*. The mutation in *E. coli* B *syn*$^-$ was later reported (Mattern *et al.*, 1966) to map at *uvrC*.

Ogawa *et al.* (1968) isolated 30 independent UV-sensitive mutants from MNNG-treated populations of *E. coli* W3623, a K12 strain from Lederberg. These were also selected on the basis that they were unable to support the growth of UV-irradiated T1 phage. Among these, 11 mapped as *uvrA* mutants, four as *uvrB*, six as *uvrC*, and three as a new class, *uvrD*. This last group, *uvrD*, is of particular interest because, unlike the *uvrA*, *uvrB*, and *uvrC* alleles which are recessive to wild type, the *uvrD* allele was reported to be dominant over wild type. *UvrE* gene mutants have been reported (see Bachmann *et al.*, 1976; Horiuchi and Nagata, 1973; Siegel, 1973*a*,*b*). One was isolated as a mutator strain, and another as a temperature-sensitive conditionally lethal mutant of a strain carrying an amber *polA* mutation and a temperature-sensitive amber suppressor. The *uvrE*$^-$ condition renders *E. coli* somewhat sensitive to UV (about two- to threefold more sensitive than its *uvrE*$^+$ parent), is a mutator allele, and is lethal when in combination with a *polA* mutation that inactivates polymerase activity but not 5′-3′-exonuclease activity (Mattern and Houtman, 1974).

1.5.2. *polA*

DeLucia and Cairns (1969) made the startling discovery that strains almost totally lacking the Kornberg polymerase, *polI*, were viable. The D_{37}, as determined by Gross and Gross (1969), in its *E. coli* W3110 genetic background, was about 2.5 J/m^2 as compared to about 100 J/m^2 for W3110. The strain was sensitive to methyl-methane sulfonate (MMS) treatment as well.

1.5.3. *lexA*

Mutants at *lexA* were reported by Howard-Flanders and Boyce (1966) to be sensitive to killing by UV, X-rays, and mitomycin C, but to be proficient as recipients during conjugation. Compared to *E. coli* with wild-type repair, *lexA* mutants degraded their DNA rapidly after UV irradiation. It was noted that *lexA* mapped near the position of the *exr* mutation in Hill's *E. coli* Bs-2 mutant and was cotransducible with *uvrA* and *met*. Witkin (1967), suggesting that *exr* and *lex* mutations

may be in the same gene, showed *exr*$^-$ mutants to be immutable by ultraviolet light. Mount *et al.* (1972) isolated two new strains mapping at *lex*, and showed *lex*$^-$ to be dominant over *lex*$^+$.

1.5.4. The *rec* Series of mutants

Clark and Margulies (1965) reported the selection from an MNNG-treated F$^-$ *E. coli* K12 population of two mutants unable to serve as females on mixing with Hfr cells. Both of these showed about 10^{-4} of the wild-type recombination frequency (although Low (1968) found no normal recombinants), as well as extremely high sensitivity to killing by UV. Revertants to wild-type UV resistance showed near wild-type recombination frequencies. Soon after, Howard-Flanders and Theriot (1966) reported the isolation of five similar mutants, all selected on the basis of sensitivity to ionizing radiation. These workers showed that all five mutants (plus one received from Clark) were sensitive to both UV (D_{37} about 0.3 J/m^2) and to X-rays, and were recombination deficient. Furthermore, such mutants, later called *recA* mutants (Willetts *et al.*, 1969), rapidly and extensively degraded their DNA after UV irradiation. The λ lysogens of such mutants showed both low spontaneous and low UV-induced production of λ phage (Brooks and Clark, 1967; Hertman and Luria, 1967). Hertman and Luria (1967) showed that in UV-irradiated *recA* λ lysogens the λ repressor was still active, whereas in UV-irradiated *rec*$^+$ lysogens the λ repressor became inactive. *RecA* mutants were found to be immutable by UV light (Miura and Tomizawa, 1968; Witkin, 1969*a*). *RecA* mapped between *cysC* and *pheA* (Willetts *et al.*, 1969).

Other recombination-deficient mutants originally described by Howard-Flanders and Theriot (1966) were subsequently mapped in a locus different from the *recA* locus of the *E. coli* map, cotransducing with *thy* (Emmerson and Howard-Flanders, 1967) and lying between *thyA* and *argA* (Emmerson, 1968). Because complementation analysis uncovered two groups of mutants mapping in this region, they were called *recB* and *recC*, mapping in the order *thyA-recC-recB-argA* (Willetts and Mount, 1969). Following initial work by Buttin and Wright (1968), mutants either in *recB* or in *recC*, but not *recA* strains, were found to have decreased levels of an ATP-dependent DNA endonuclease (Oishi, 1969; Barbour and Clark, 1970; Goldmark and Linn, 1970). *In vivo* complementation between *recB* and *recC* strains was found to give rise to normal levels of ATP-dependent nuclease activity (Barbour and Clark, 1970). The enzyme has an associated DNA-

dependent ATPase activity (Nobrega *et al.*, 1972) that hydrolyzes eight or nine ATP molecules per DNA phosphate chain break *in vitro*. The *recB* and/or *recC* phenotype was found to be suppressed by mutations occurring either at the *sbcA* or *sbcB* loci (Barbour *et al.*, 1970; Kushner *et al.*, 1971). In both cases, suppression was accompanied both by increased recombination frequency and by increased resistance to mitomycin C. In all *sbcB*-suppressed mutants, exonuclease I was found to be deficient (Kushner *et al.*, 1971). The *sbcA* mutants contained an increased level of an ATP-indepent DNase (Barbour *et al.*, 1970). However, *E. coli* K12 strains belonging to the AB1157 series of mutants apparently lacked the *sbcA* locus; therefore, no *sbcA* revertants of this series have been observed (Barbour *et al.*, 1970). Another class of *recBC* suppressor revertants, due to additional mutations at *xonA*, was found to be characterized by wild-type sensitivity to either UV or mitomycin C treatment, by defective exonuclease I, and by the same low recombination frequency characteristic of the original *recB* or *recC* strains (Kushner *et al.*, 1972), showing that defective repair of UV damage in *recB* and *recC* strains is phenotypically separable from defective recombination. The DNase whose activity is increased in *sbcA* suppressor revertants has been purified and named exonuclease VIII (Kushner *et al.*, 1974). It is of interest here to point out that λ phage mutant at *red* (recombination deficient, either at *exo* or *bet*) and *gam* cannot grow in *recA* strains of *E. coli*. Rare plaques on *recA* strains have been selected and studied. Such phages were called λ reverse (λ rev) and were found to have acquired a host gene coding for an enzyme with properties very similar to exo.VIII. Furthermore, λ *rev*, unlike λ *red gam*, expresses a recombination function (Gottesman *et al.*, 1974).

Further rec^- mutant isolation studies by Horii and Clark (1973), using as parents the $recBC^-$ $sbcB^-$ strains which were phenotypically rec^+, revealed that rec^- mutants in any one of several different genes could be isolated. *RecF* and *recL* are discussed here. Although *E. coli* wild type with respect to repair and *E. coli* carrying mutations both at *recBC* and *sbcB* have about the same sensitivity to inactivation by UV, the introduction of the $recF^-$ allele into the latter decreased the UV resistance to a level similar to that seen in *E. coli* mutant only at *recA* (their D_{37} being about 0.6 J/m^2), while introduction of the *recF* gene into *E. coli* with wild-type repair does not have such a drastic effect (D_{37} about 9 J/m^2).

RecL mutants obtained from *recBC sbcB* parents are about as UV sensitive as the corresponding *recF* mutants. Rothman and Clark (1977*a*,*b*) and Rothman (1978) have shown that pyrimidine dimer exci-

sion is normal in a *recF* strain, but is slow in *recL* strains. Further, while the average repair patch size was 30 nucleotides in strains with wild-type repair, it was found to be 360 nucleotides in a *recL* strain. It is important to emphasize that there is good evidence that mutations *uvrD*, *uvrE*, and *recL* are mutations in the same gene (Kushner *et al.*, 1978).

1.5.5. *lig*

Pauling and Hamm (1968) isolated a temperature-sensitive, UV-sensitive mutant, *ts-7*, that accumulated short nascent DNA fragments at the nonpermissive temperature. The mutant was found to have a defect in DNA ligase (Pauling and Hamm, 1969*a*,*b*), and NAD-requiring enzyme that joins 3′-OH–5′-PO_4 nicks in double-stranded DNA (Gellert, 1967).

1.5.6. *rho*

E. coli strains having mutations at *rho* (required for termination of transcription) conferred both temperature-sensitive and UV-sensitive phenotypes. Such mutants were isolated by their ability to suppress polar mutations (Das *et al.*, 1976).

2. λ PHAGE

Much UV work has been done using λ phage of *E. coli*, discovered and named by Esther Lederberg (1951). She noticed that *E. coli* K12 cultures produced plaques on lawns of a UV mutant (W518) which had evidently lost its λ prophage, thereby becoming sensitive to infection by the phage, which was liberated by spontaneous lysis of wild-type K12 cultures. A map of λ was published by Echols and Murialdo (1978).

2.1. UV Induction of λ Phage

2.1.1. Direct Induction

Inspired by Lwoff's discovery (Lwoff, 1951, 1953) that UV-irradiated *B. megaterium* produce phage on postirradiation incubation,

the UV induction of the vegetative state of λ phage in λ lysogens was studied quantitatively by Weigle and Delbrück (1951). A UV dose of 24 J/m² delivered to *E. coli* K12 (λ) induced about 95% of the bacteria to produce on the average 130 pfu/cell after 2 hr of incubation. Reflecting intracellular phage growth, cells increased considerably in volume prior to lysis. The mutation λ *ind*⁻ resulted in UV being ineffective in inducing λ from strains carrying λ *cIind*⁻ as a prophage (Jacob and Campbell, 1959). λ *ind*⁻ is dominant over λ⁺, and maps at the *cI* repressor gene.

2.1.2. Indirect Induction

Borek and Ryan (1958, 1960, review 1973) found that the lysogenic bacterium itself did not need to be irradiated to induce phage production, but that the introduction of an *F* factor from an irradiated nonlysogenic donor into a nonirradiated F^- λ lysogen gave good induction of the recipient prophage. This phenomenon was termed "indirect induction."

Later, Devoret and George (1967) showed that, at optimal doses, the efficiency of indirect induction was 50% that of direct induction, and that both processes occurred with two hit kinetics: 30 J/m² delivered to the donor gave 100-fold more indirect induction than did 3 J/m². Furthermore, while indirect induction was found to occur in the case of F^+ (irradiated) × F^- (λ^+), confirming the results of Borek and Ryan (1958), it was not found in the case of *E. coli Hfr* (UV-irradiated) × F^- (λ^+). Rosner *et al.* (1968) pointed out that only replicons capable of being stably inherited without integration (*F*, *Col*I, *RTF*, P1) appeared to be able to mediate indirect UV induction of λ. These authors showed that irradiated P1 phage, on infecting an *E. coli* K lysogenic for λ, 434, or 21, could cause the induction of all these resident prophage. Attempted indirect induction by infecting λ-lysogens with UV-irradiated phages 434hy, θ80, or T6 was unsuccessful. Further, UV-irradiated P1 could not cause induction of *ind*⁻ lysogens or of *recA* λ lysogens. Induction of λ by infection of a lysogen with irradiated P1 was prevented when the λ lysogen was also lysogenized by P1, an effect that was also found when *F* replaced P1 as both the inducer and resident replicon. However, irradiated P1 caused induction when infecting an F^+ λ lysogen, showing that replicon protection against induction only occurs when the protective resident replicon is the same as the irradiated inductive replicon. The authors suggested that "the functions which permit a replicon to cause indirect induction may also be

involved in coordinating its replication with that of the host chromosome."

2.1.3. Dependence of Direct Induction on DNA Repair Functions

Using *E. coli* K12S *hcr*$^-$ (λ) (Harm, 1965) and using *E. coli* λ lysogens carrying the *dar1*, *dar2*, *dar3* and *dar4* mutant alleles, Mattern *et al.* (1965) showed that less UV exposure is required to induce the productive assembly of λ in repair-deficient (all *uvr*) *E. coli* strains than in their repair-proficient parents. Both laboratories reported maximum λ induction at doses of about 40 J/m^2 to repair-proficient cells, while in the repair-deficient cells, such doses varied, Harm reported maximum induction at 2 J/m^2, Mattern *et al.* at about 7 J/m^2. The difference could have been due to the different media and techniques employed, because Mattern *et al.*, using Harm's strains, obtained results consistent with those given by their own *dar* mutants. A *polA* mutant and a *polA uvrA* double mutant were found to be induced with the same rapid dose–response kinetics characteristic of a *uvrA* mutant or a *uvrA polA* double mutant (Monk *et al.*, 1971). Thus UV induction of λ appeared to be due to postirradiation biochemical processes operating on DNA containing repairable damage. Brenner and Groman (1966) demonstrated the role of the *cI* gene in maintaining the lysogenic state: UV irradiated λ lysogens produced λ phage on superinfection by λ *cI* (clear plaque, mutant in the *cI* repressor gene) but such induction was suppressed on superinfection by λ$^+$.

By elegant biological methods, Tomizawa and Ogawa (1967) showed intracellular λ repressor to be destroyed after UV irradiation by a process that was blocked by KCN or chloramphenicol. The capacity to destroy the λ repressor could be induced by a 30-min incubation of a suspension of UV-irradiated nonlysogens, and, further, the ability to destroy λ repressor could not be induced by UV irradiation of *rec*$^-$ (*recA*, Miura and Tomizawa, 1968) bacteria. This fact helped to explain the finding of Hertman and Luria (1967), who were unable to induce λ after UV irradiation of *recA* lysogens, but could "rescue" the induction by P1 transduction of the *recA*$^+$ gene into irradiated *recA*$^-$ lysogens. Brooks and Clark (1967) also reported a *rec*$^-$ λ$^+$ lysogen to be noninducible by UV, but that a *rec*$^-$ λ *cI*857 lysogen showed normal thermal induction when compared to *rec*$^+$ λ *cI*857. The results of these studies showed that, while *recA*$^-$ mutants can support the growth of λ, they are unable to destroy the *cI* repressor after UV induction, so that,

after attempted UV induction of a *recA* lysogen, the biochemistry of phage growth is not initiated. Donch *et al.* (1970, 1971) found *exrA* λ-lysogens to be UV induced only to low levels of λ production and that such levels were attained only at prolonged incubation times. Both *exrA* λ *cI*857 and exr$^+$ λ *cI*857 lysogens were thermally induced with similar kinetics. Thus *exrA* strains, like *recA* strains, are able to support λ production, but cannot destroy λ repressor.

2.1.4. Dependence of Indirect Induction on DNA Repair Functions

George and Devoret (1971) irradiated (40 J/m^2) a *uvrB* F^+ strain and found it to be a tenfold more efficient donor than its *uvr*$^+$ derivative in inducing λ when mated with a *uvr*$^+$ F^- λ-lysogen. A donor mutation at *recA* was without effect on indirect induction kinetics, but a *uvrA recA* donor was a more efficient inducer than an *Flac*$^+$ strain with wild-type DNA repair. When DNA repair-deficient λ lysogens were used as recipients in indirect induction experiments, *recA* lysogens were not induced, but *recB* and *uvrB* lysogens (George and Devoret, 1971) and *uvrA* lysogens (Benbow *et al.*, 1973) behaved as did their repair-proficient counterparts.

Thus a repair-deficient donor (which did not repair the *F* DNA before transfer) was more effective than a repair-proficient donor in inducing λ from a recipient lysogen. The repair state of the recipient was of no importance as long as λ could be induced from it by direct induction methods. Thus, following Benbow *et al.* (1973), the induction is probably initiated by products arising during the synthesis of the DNA that accompanies transfer, during which the incoming damaged *F* DNA strand is used as a template.

That the molecular initiator of induction could be strand breaks, repair, or DNA synthetic intermediates was suggested by the *in vitro* work of Sussman and Ben Zeev (1975). They found that the λ$^+$ repressor protein (but not that from λ *ind*$^-$) was removed from λ DNA in the presence of DNA extracted from *E. coli* cultures that had been treated prior to DNA extraction in various ways (thymidineless conditions, UV followed by incubation, mitomycin C treatment, and high-temperature incubation of a *ts* ligase mutant) known to induce the initiation of λ production *in vivo*. DNA from untreated cells did not compete for the λ repressor.

In support of this hypothesis, Braun (1976) published data in support of the corollary that no induction should occur if no such DNA

metabolic intermediates were allowed to accumulate. Using a *dnaA uvrB* λ lysogen preincubated at 42°C for 1 hr (the dnaA mutation used allowed elongation but conferred temperature-sensitive initiation), Braun showed that UV produced no induction of λ in a culture maintained at 42°C.

If lysogens mutant at either *dnaA* or *uvrB* were used in the same experiment, or if the *dnaA uvrB* lysogen was irradiated at 34°C and then shifted to 42°C, production ensued. The controls are consistent with the finding of Lydersen and Pollard (1977) that λ repressor destruction occurred as rapidly in the presence of hydroxyurea as without. Roberts and Roberts (1975) showed, using antiserum against the λ repressor, that, after UV induction, wild-type λ repressor molecules (but not the *ind*$^-$ repressor) were cleaved, the postirradiation half-life of whole repressor molecules being about 20 min.

2.1.5. "*tif*" and Protein X

Goldthwait and Jacob (1964) isolated T44, later called "*tif*" (for *t*hermally *i*nduced *f*ilamentation), a mutant of *E. coli* C600 (λ) that formed colonies at 30°C, but not at 40°C. Although the mutation was in the bacterial (not the prophage) chromosome, λ was induced at 40° and proliferated vegetatively, showing that T44 was defective in a host function that stabilized the prophage state. The addition of cytidine plus guanosine inhibited the thermal induction, while adenine accelerated it. T44, even cured of its prophage, formed filaments and died at 40°C (Kirby *et al.*, 1967), effects shown after UV by *E. coli* B or *lon*$^+$ (*fil*$^-$) mutants of *E. coli* K12. Revertants of T44 (λ) able to grow at 40°C did not form filaments and fell into two classes. Class I mutants behaved like *recA* mutants in their low spontaneous induction of λ, their UV-sensitive colony-forming ability, their reduced recombination frequency (less than 1% of C600 (λ)) when mated with an appropriate Hfr, and their wild-type host cell reactivation of UV-irradiated λ and T3 phage. Class II appeared to be due to reversions to wild type.

Protein X (and protein Y) were identified in gel patterns of membrane proteins by Inouye and Guthrie (1969). An *E. coli* mutant, temperature sensitive in its DNA synthesis, showed (compared to a revertant) a decrease in protein Y (about 34,000 molecular weight) and an increase in protein X (about 39,000 molecular weight) on incubation at 41°C. The amounts of proteins X and Y in the mutant did not change relative to those present at 28°C. Pulse chase experiments

(Inouye and Pardee, 1970) showed neither protein to be the precursor of the other. The rate of protein Y synthesis was decreased relatively slowly in cells blocked in DNA synthesis, while protein X appeared rapidly. Inouye (1971) found that increased protein X synthesis also occurred if a thymine-requiring mutant were starved for thymine. (Thymine starvation of λ lysogens results in induction.) In a starved *recA thy*$^-$ strain, however, no protein X was produced. Gudas and Pardee (1975, 1976; see also Gudas, 1976) reported that naladixic acid treatment of repair proficient *E. coli* B and *E. coli* K12 resulted in the production of large amounts of protein X, which, in amount, increased to 3–4% of the total cell protein within 1 hr of treatment. (Naladixic acid is a good inducer of λ phage and is a specific inhibitor of DNA gyrase. See Sugino *et al.*, 1977, and Gellert *et al.*, 1977.) Naladixic acid treatment of *recA* (including *recA*56), *recB*, *recC*, *recA recB*, *lex-3*, or *exrA* mutants did not induce protein X production, whereas a strain mutant at *tif* produced high amounts of protein X at 41°C without naladixic acid treatment. An UV-resistant, temperature-sensitive derivative of *lex-3* (*lex-3 tsl*$^-$, Mount *et al.*, 1973; *tsl* mapped very close to *lex*) and a *recA* derivative of this (*lex-3 tsl*$^-$ *recA*56) both produced protein X at 41°C, also without naladixic acid induction. The result showed that *recA56* was mutant in a regulatory (not necessarily a structural) gene. In the presence of 4 mM Mg^{2+}, protein X bound to single-stranded better than to double-stranded DNA (Gudas and Pardee, 1975). Little and Hanawalt (1977) showed that UV also induces the production of protein X in *E. coli*, and, further, that *recB* strains are induced by UV to produce protein X, this latter unlike the result of Gudas and Pardee (1975) using naladixic acid as the inducer. Further, these workers showed that DNA fragments were not enough to induce protein X since λ phage grown in *E. coli* C (whose DNA is rapidly nicked and degraded on injection into *E. coli* K strains) does not induce protein X in these strains.

McEntee *et al.* (1976) studied the product of the *recA* gene by infecting heavily UV-irradiated cells with λ *precA* (a plaque-forming specialized transducing phage), labeling the proteins synthesized, and then subjecting these to gel electrophoresis. By the use of various *recA* genes in the λ*precA* phage, the *recA*$^+$ protein was identified as a multimeric protein containing a single polypeptide of 40,000 molecular weight. The fact that the molecular weight of the monomeric form of the *recA* protein was very similar to published values of the molecular weight (in SDS gels) of protein X suggested the possibility of their being one and the same protein. Indeed, four groups (McEntee, 1977;

Gudas and Mount, 1977; Emmerson and West, 1977; Little and Kleid, 1977) provided strong evidence that protein X is the product of the *recA* gene. The function of protein X was made clearer when Roberts *et al.* (1977) reported that a preparation, 95% of which was protein X, cleaved the λ repressor in a reaction that required ATP. Following this, Roberts *et al.* (1978) reported that purified protein X could carry out the reaction. Furthermore, a *tif*$^-$ *recA* protein was fivefold more active per molecule than the wild-type *recA* protein, explaining in part the behavior of *tif* mutants. Further, *recA* protein, in the presence of ATP, produced D loops (localized regions of unwinding) in double-stranded DNA. The reaction was stimulated by single-stranded DNA or oligonucleotides which did not need to be homologous to the DNA unwound (Cunningham *et al.*, 1979).

In studying the properties of *lex* mutants, Mount (1977) prepared a series of mitomycin-C-resistant revertants starting from the mitomycin-C-sensitive strain DM1180 (*lexA*3 *tif*-1 *sfiA*). From these, DM1187 was chosen as representative of a class of revertants having at the *lex* site an additional mutation called *spr* and giving rise, at either 30° or 40°C, to clear plaques when infected with λ$^+$ but to turbid plaques when infected *cI ind*$^-$. Such behavior suggested that DM1187 constitutively produced an inactivator of the *cI* repressor. Indeed, DM1187 was found to produce the *recA* product, protein X, at constitutive levels (Gudas and Mount, 1977). Gudas and Mount (1978) isolated DM1285 as a phenotypic revertant of DM1187 which formed turbid plaques on infection with λ$^+$. However, both strains (but not JM1 (*lex*$^+$ *tif*$^+$ *sfi*$^+$) wild type) gave clear plaques with λ *cI ind*^{s}, whose repressor was believed to have decreased affinity for the operator (Lieb, 1964). The mutation responsible for the difference between DM1295 and DM1187 maps at *lexA* and shuts off constitutive protein X production. The protein X made on mitomycin C treatment of DM1295 appears to be the same altered protein X (*tif*) as that made by DM1187, a fact consistent with the mapping data, but is induced poorly compared to the induction seen in the JM1 strain, which has wild-type repair. After naladixic acid treatment (which blocks DNA gyrase), DM1295 showed a burst of protein X synthesis in contrast to the prolonged synthesis induced by naladixic acid in JM1.

2.1.6. Protease Inhibitors and the Induction of λ Phage

Meyn *et al.* (1977) obtained biological evidence implicating a protease in the induction of phage by showing that a protease inhibitor,

antipain, blocked the thermal induction of λ^+ phage that occurs in a *tif* lysogen, but did not block thermal induction of a λ *cI*857 *ind*$^-$ *tif* lysogen—a situation in which the repressor itself is temperature-sensitive. Radman *et al.* (1977) presented very similar data for the protease inhibitor TLCK. Meyn *et al.* (1978) extended their observations to include elastatinal, TLCK, and DFP, but showed that other protease inhibitors, leupeptin, chymostatin, TAME, and TGCK, are not effective in blocking the induction of λ phage.

Thus the picture we might have of the events leading from UV damage to cleavage of the lambda repressor in a wild-type lysogen might be like this (thanks to McEntee, 1977; Gudas and Mount, 1977): After UV damage is introduced into the lysogen's DNA, abortive attempts at DNA synthesis, which are more frequent in cells lacking *uvr* or *polA* functions, generate altered substrate (primer, fork, etc.) which would also be generated in the presence of gyrase inhibitors. Such attempted DNA synthesis produces DNA nicks or gaps to which the λ repressor is drawn. By binding some diffusible molecule such as adenine or adenosine, molecules of protein X (present at a low level) attain a conformation such that protein X is now active and is able to cleave molecules for which it is specific. Because *lex* (or *exr*) mutants give rise to only small amounts of λ on UV induction, it would be convenient if the *lexA* product were both the repressor of the *recA* gene operator and cleavable by the activated protein X. Thus activated protein X would cleave the *lexA* repressor of the *recA* gene, leading to the full-blown induction of protein X which, when activated, would lead to rapid irreversible cleavage of the remaining repressor molecules. The function of protein Y is unknown, but its noted disappearance is consistent with idea that it is cleaved by protein X.

2.2. Host Cell Reactivation (Hcr)

Ellison *et al.* (1960) first reported the phenomenon now called host cell reactivation (Hcr). They observed that the survival of UV-irradiated T1 phage was strongly dependent on the cell strain used in the phage assay: Whereas about 100 sec of UV was required to inactivate T1 to 1% survival using *E. coli* B in the phage assay, only 20 sec was needed if *E. coli* Bs-1 were used. The phenomenon was interpreted as indicating that *E. coli* Bs-1 lacks a gene function essential for the growth of UV-irradiated phage. Because Setlow and Carrier (1964) found that *E. coli* Bs-1, unlike its parent *E. coli* B, lacked the ability to remove UV-

produced thymine dimers from its DNA, and because of the known extreme sensitivity of *E. coli* Bs-1 itself to inactivation by UV, the cell-strain-dependent survival of UV-irradiated phage T1 probably reflected cell-strain differences in ability to repair UV-damaged phage DNA. Host cell reactivation, then, is observed if the survival of a damaged population of viruses is a function of the cell strain infected.

2.2.1. *uvr* and *rec* Mutants

2.2.1a. Biological Effects

Harm (1963*a*) and Rorsch *et al.* (1963) first reported that host cell reactivation occurs when UV-irradiated λ phage were assayed on various hosts. Harm reported that about 6.4-fold more UV light was needed to inactivate λ phage to a given survival when the phage was assayed in the wild-type strains, *E. coli* K12S and *E. coli* C, than if UV-sensitive mutants (*E. coli* K12S *hcr*$^-$ and *E. coli* C *syn*$^-$) were used (Fig. 1; see Section 1.5.1). Soon after, Mattern *et al.* (1965) published an impressive paper comparing *E. coli* strains, each carrying one of the six recently isolated *dar* genes ("*dar*" for "*dar*k *r*eactivation deficient"), with the parent of these strains, KMBL49. Of these, bacteria carrying *dar*l, *dar*3, *dar*5, and *dar*6 were as host cell reactivation deficient as the K12 *hcr*$^-$ of Harm (1963*a*). Bacteria carrying *dar*2 or *dar*4 were much less deficient in host cell reactivation, giving rise to an inactivation slope of UV-treated phage about twice as steep as that of the parent.

Ogawa *et al.* (1968) reported UV survival curves of λ phage infecting *E. coli* K12 strains both wild-type with respect to repair and mutant at each of the *uvr* loci known at that time: *uvrA*, *uvrB*, *uvrC*, and *uvrD*. The *uvr* mutants showed decreased host cell reactivation of λ phage, a 44 J/m^2 dose resulting in about 20, 0.6, 0.15, 0.03, and 0.02% on wild-type and *uvrC*, *uvrD*, *uvrA*, and *uvrB* strains, respectively. The curves showed two-to-four-hit shoulders. The respective D_{37}'s of the λ phage are about 27, 8.6, 6.8, 5.4, and 5.2 J/m^2. Calculation (1.4.) shows that about three pyrimidine dimers produced per λ genome correlate with one lethal hit, taking the data from the most sensitive case, that obtained with the *uvrB* strain. In the wild type, the production of about 12 pyrimidine dimers corresponds to a lethal hit.

Using an *E. coli* strain mutant at *uvrE*, Siegel (1973*a*) found that the slope of the survival curve of UV-irradiated λ vir was not more than twofold steeper than when the wild-type strain was used, agreeing with

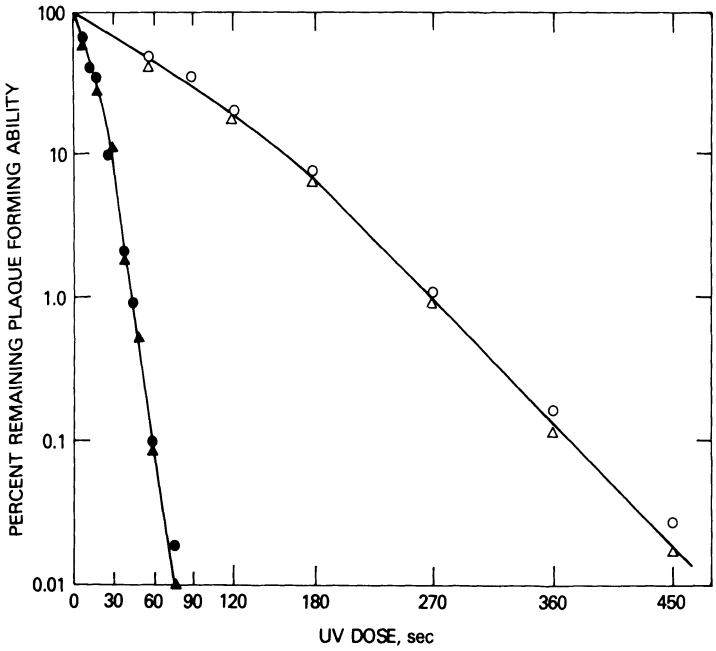


Fig. 1. Survival of λ phage as a function of (preinfection) UV dose using repair-proficient and repair-deficient hosts. Dose rate was about 0.6 J/m²/sec. (o, *E. coli* K12S; Δ, *E. coli* C; ●, *E. coli* K12S *hcr*⁻ (*uvrA*); ▲, *E. coli* C *syn*⁻ (*uvrC*). Redrawn from Harm (1963*a*), with permission.

the result of Horiuchi and Nagata (1973). Similar sensitivity was judged for *polA* mutants (Ogawa, 1970; Klein and Niebch, 1971; Rothman and Clark, 1977*a*) and a *recL* mutant (Rothman and Clark, 1977*a*). Two *recF* mutants showed wild-type levels of *hcr* (Rothman and Clark, 1977*b*).

Radman *et al.* (1970) performed UV survival curves of lambda phage, using as hosts various strains of *E. coli* having combinations of *uvrA* and *recA* mutations (see Fig. 2). Survival curves of irradiated λ phage obtained by infecting *uvr*⁻ hosts indicated far more UV sensitivity than those obtained by infecting *uvr*⁺ hosts, showing first either a slight or no shoulder, then an exponential decline. The phage and host recombination functions play some role in the repair of UV-damaged λ phage in *uvr*⁻ bacteria, acting to decrease the slope about twofold, i.e., making the UV dose twofold less lethal or repairing half the UV-produced damage. About two dimers per phage genome correlated with one-hit inactivation under the most sensitive conditions (phage red⁻, *E. coli uvrA recA*).

The final slopes of all curves obtained using uvr⁺ bacteria were about fivefold less steep than those obtained using the corresponding

uvr$^-$ bacteria. If the uvrA product were functional but the host *recA* and phage *red* genes were nonfunctional, successful hostcell reactivation occurred in only 15% of the infected cells (the extrapolation of the high dose portion of the curve defined by the open triangles back to zero dose intercepted the axis at about 0.15). When both recombination systems were functional, however, repair of λ phage was evident in all infections.

Fornili *et al.* (1971) proposed an interesting model to account for such survival kinetics. The model is interesting because it goes outside classical "target theory" by hypothesizing a target that changes its properties with dose. The model goes as follows: (1) repair of damaged

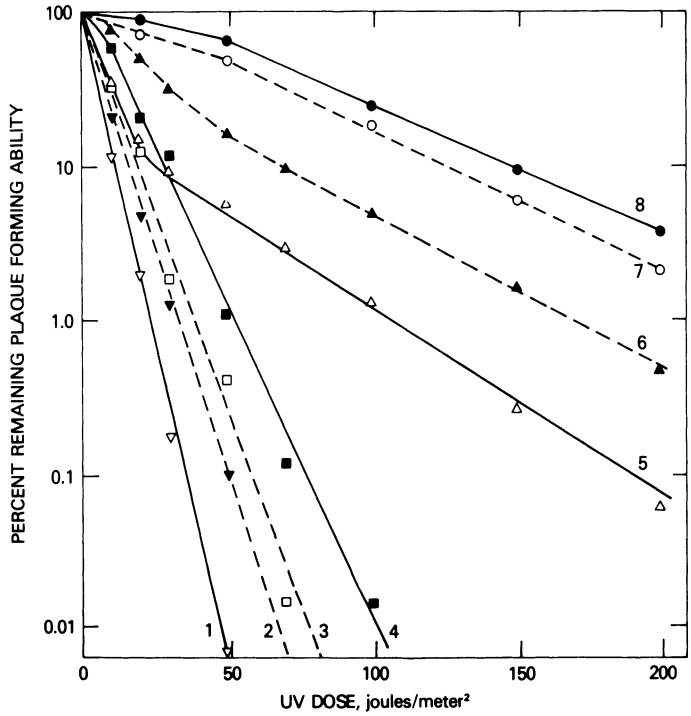


Fig. 2. Survival of *red*$^+$ and *red*$^-$ phage λ as a function of UV dose, using *uvr*$^+$ *rec*$^+$ (AB1157), *uvr*$^+$ *recA* (AB2463), *uvrA rec*$^+$ (AB1886), and *uvrA recA* (AB2480) *E. coli* strains as hosts.

Curve	λ	Host	Curve	λ	Host
1	*red*$^-$	*recA uvrA*	5	*red*$^-$	*recA uvr*$^+$
2	*red*$^+$	*recA uvrA*	6	*red*$^+$	*recA uvr*$^+$
3	*red*$^-$	*rec*$^+$ *uvrA*	7	*red*$^-$	*rec*$^+$ *uvr*$^+$
4	*red*$^+$	*rec*$^+$ *uvrA*	8	*red*$^+$	*rec*$^+$ *uvr*$^+$

Redrawn from Radman *et al.* (1970) with permission.

phage by *uvr*-dependent excision can be effective only prior to λ DNA synthesis; (2) phage repair by *recA*-dependent processes is effective only during or after DNA synthesis; and (3) only above a certain UV dose is λ DNA replication inhibited for a period long enough to allow *uvr*-dependent excision repair to occur. Following the analysis of the curves, the size of the region in λ DNA in which UV damage can inhibit λ DNA replication is 13% of the genome length.

The prediction (based on the preceeding hypothesis) that UV-damaged λ, blocked in DNA synthesis, should survive better in uvr^+ rec^- hosts than does UV-damaged λ uninhibited in DNA synthesis was investigated. Using UV-treated λ cI O_{28}, temperature sensitive in DNA synthesis, Radman *et al.* (1970) infected uvr^+ rec^+, uvr^- rec^+, uvr^+ rec^-, and uvr^- rec^- cells at 42°C. After incubating the complexes at 42°C for various times before plating, it was found that, as expected, blocking DNA synthesis preferentially increased survival of λ in uvr^+ hosts. Qualitatively, the data supports the hypothesis, but a more thorough study should be done.

Kneser (1968) had reported data similar to that of Radman *et al.* (1970), but his data were not as extensive or as clearly interpreted. He (Kneser, 1966) had found caffeine (0.1%, incorporated in the plating medium) to mimic the effect of a uvr mutation (see also Metzger, 1964), reducing the survival of irradiated λ greatly when infecting a uvr^+ rec^+ or uvr^+ rec^- host.

2.2.1b. Pyrimidine Dimer Excision

Boyle and Setlow (1970) reported that pyrimidine dimers were excised from the DNA of λ phage (m.o.i.~1) infecting AB2497 (thy^- hcr^+) hosts more efficiently than when infecting AB2500 thy^- hcr^- hosts, thereby correlating *hcr* with dimer excision. Chloramphenicol did not greatly affect the excision rate, showing that excision of dimers from a UV-damaged genome did not depend on prior protein synthesis.

2.2.1c. DNA Circularization

Tokunaga (1975), using a *uvrB* host, showed that 167 and 330 J/m^2 of UV delivered to λ phage reduced the normal conversion of the injected λ DNA to supertwisted circles to about 25% and 10% of that of the nonirradiated phage. Excision played little role in this phe-

nomenon because a *uvrB* host was used. Because the multiplicity was 3, however, recombination mechanisms may have kept some of the λ DNA circles open.

In investigating UV-photoproduct-specific nicking of phage λ DNA, Seeberg and Johansen (1973) prepared λ *cI*857 *ind*$^-$ lysogens of an AB1157 series of *uvr* mutants [AB1886 (*uvrA*), AB1885 (*uvrB*), and AB1884 (*uvrC*)] as well as of AB1157 (*uvr*$^+$). Such lysogens neither support the growth of superinfecting λ (the infection does not proceed further than the formation of supertwisted circles) nor are they induced by UV to produce phage since the prophage carries the *ind*$^-$ mutation (UV inductionless) at the *cI* gene. The lysogens were first irradiated with 10 J/m^2 (to induce any enzymes that might play a role in repair), then infected (m.o.i. = 3–5) with ^{3}H λ *cI*857 *ind*$^-$ phage, chilled in ice, and again UV irradiated. Finally, they were incubated in chloramphenicol at 42°C to block any loss of the supertwisted λ circles that would have occurred in the absence of chloramphenicol. In AB1157 lysogens treated as above, the λ supertwisted DNA was nicked very rapidly ($t_{1/2}$ about 20 sec) on heating to 42°C. In *uvrA* or *uvrB* lysogens, there was no nicking, whereas such nicking by *uvrC* lysogens was slow, requiring 5 min to nick to the same extent that *uvr*$^+$ lysogen required 20 sec to accomplish. In a *polA*1 lysogen, no λ circles were found after 20–50 sec of incubation, giving the impression that *polA* function is required to seal the nicks introduced by *uvr*$^+$ repair functions. The UV dose to the infected cells required to observe maximum nicking was 60 J/m^2, a dose which would produce on the order of 40 pyrimidine dimers per λ circle. Thus either the observed nicking occurred at nondimer sites or a dimer-specific nick that occurs during repair has a one in 40 chance of being detected due to rapid sealing by other enzymes (*polA*?).

2.2.2. Infectious DNA Assays

Several groups of investigators have measured the inactivation of λ by assaying survival using infectious DNA assays. Thus Ogawa and Tomizawa (1973) found that the survival of plaque-forming ability due to λ DNA extracted from UV-irradiated virions (measured using *uvr*$^+$ or *uvrA* hosts) was identical to that obtained when the same treated virions were assayed by the conventional plaque assay on these strains. Thus strong evidence was provided for the idea that UV-damaged viral structural proteins play little role in determining the survival of the

phage. Furthermore, λ*cI* DNA extracted 90 min after ^{15}N-labeled λ*ind*$^-$ (*rec*$^+$) lysogens were infected with UV-irradiated (light) *cI* phage was found unrepaired (by λ spheroplast assay using Hcr deficient hosts) if the lysogen carried mutations at *uvrA*, *uvrB*, or *uvrC*, but repaired if the lysogen were *uvr*+. Because the λ *cI* genome will not replicate even once when infecting lysogens (Wolf and Meselson, 1963), the observed repair of the λ DNA did not require DNA synthesis.

Wackernagel (1974) found the survival of UV-irradiated λ DNA to be much like that of UV-irradiated phage when Hcr$^-$ hosts were used in a spheroplast assay, but Hcr$^+$ spheroplasts provided slightly reduced (20%) survival of UV-irradiated DNA compared to the survival of UV-irradiated phage infecting Hcr$^+$ cells. This is at some variance with the result of Ogawa and Tomizawa (1973) and may reflect differences in techniques used to prepare spheroplasts. It is possible that some *uvr* enzymes were either inactivated or leaked out of Wackernagel's spheroplasts, since his assay of the same UV-irradiated DNAs using the calcium technique (Mandel and Higa, 1970) to infect Hcr$^+$ hosts resulted in as efficient restoration of plaque-forming ability as obtained for UV-irradiated whole phage infecting untreated Hcr$^+$ cells. Breseler *et al.* (1974) reported somewhat similar data on the Hcr effects on UV-irradiated λ phage and λ DNA. Tomilin and Mosevitskaya (1975) reported that they were able to treat UV-irradiated λ DNA with an enzyme preparation from *M. luteus* which produces nicks near pyrimidine dimers (see Riazuddin and Grossman, 1977*a*,*b*), and thereby increases its infectivity (calcium technique) when infecting *E. coli uvrA*, *uvrB*, or *uvrC* mutants, but not when infecting the *uvr*$^+$ AB1157 strain. This would mean that the *M. luteus* preparation can substitute *in vitro* for the *in vivo uvrA*, *B*, and *C* functions.

2.2.3. Other Host Cell Reactivation Studies

Other results of interest concerning Hcr include those of Geissler (1970) that the λ-like phage, 434, when UV-irradiated shows behavior similar to that of UV-irradiated λ when infecting *hcr*+ *rec*+, *hcr*+ *rec*−, *hcr*− *rec*+, and *hcr*$^-$ *rec*$^-$ *E. coli* hosts.

Bridges (1975) reported that one of the mutants of Bonhoeffer (1966), temperature-sensitive in its DNA synthesis, showed deficient host-cell reactivation of UV-irradiated lambda phage at 34°C (the nonlethal temperature), but normal post-UV survival of colony-forming ability at 34°C. Bridges suggested that UV photoproducts might block

DNA synthesis in the irradiated bacterium, allowing time for complete repair before DNA synthesis resumed. He found a 60-min adsorption time in 0.01 M $MgSO_4$ to result in increased survival (with respect to that obtained at shorter adsorption periods) of UV'd λ phage infecting the mutant, a finding which he believed supported his suggestion. However, no control with *E. coli* with wild-type repair was done, nor were studies using λ having temperature sensitive DNA synthesis.

Suzuki *et al.* (1969) reported finding a mutant (*urt43*) of *E. coli* C600 which was temperature sensitive with respect to post-UV colony-forming ability, but which showed temperature-independent colony forming ability if not irradiated. At 42°C, the UV inactivation slope was about tenfold steeper than at 25°C (which was the same as that at 30°C), although, even at these latter temperatures, the mutant was UV sensitive with respect to the parent. If UV-treated *urt43* were incubated at 30°C in minimal medium with the required amino acids prior to plating, its UV survival increased dramatically on plating. However, *urt43* performed little host-cell reactivation of UV-irradiated lambda phage at either temperature. The increase was blocked in the absence of required amino acids or if chloramphenicol were present during incubation. More will be said about *urt43* in Section 2.5.

2.3. Multiplicity Reactivation

Multiplicity reactivation was first observed as an increase in titer of a UV-irradiated (or otherwise damaged) virus preparation as the m.o.i. was increased. The reactivation was found to be due to the interaction of otherwise inactive phage particles (Luria, 1947; Luria and Dulbecco, 1949). The phenomenon was observed to occur strongly in the case of T2, T4, T5, and T6 phages, to a lesser extent with T1 phage, and not with T7 phage. In noting λ phage not to be subject to multiplicity reactivation (see the citation of ref. 4 in Weigle, 1953), Jacob and Wollman (1955), having presented data showing that recombination between UV-irradiated λ phages does indeed occur, suggested that

> It could, at first sight, appear surprising that a bacteriophage in which irradiation markedly increases the recombination frequency manifests no multiplicity reactivation. To explain this apparently paradoxical situation, one can imagine either that multiplicity reactivation results from a physiological mechanism and not from a genetic one as it was suggested to be by Dulbecco, or that the lesions produced by UV irradiation, which, in virulent phage are restorable by multiplicity reactivation, can be restored by the bacteria themselves in the case of λ. [Author's translation.]

Their second suggestion was, of course, correct, anticipating the report (Hill, 1958) of UV-sensitive mutants by 3 years.

Although multiplicity reactivation was not initially observed in phage, Kellenberger and Weigle (1958) did observe a small effect using very high multiplicities. Later, Baker and Haynes (1967) showed multiplicity reactivation of UV-irradiated λ phage to be much more easily observable if a *uvr* rather than a wild-type strain were multiply infected with the damaged phage. Evidently, the difficulty encountered earlier in observing such reactivation of λ phage was due to the extensive *uvr*-dependent repair either competing with or masking effects due to increased multiplicities. The absence of excision repair allowed multiplicity reactivation to stand out. Huskey (1969), using a recombination-deficient λ/φ80 hybrid phage and *uvrA*, *recA E. coli* mutants, showed that multiplicity reactivation depends almost entirely on a functional recombination system—either phage or bacterial encoded.

2.4. UV-Enhanced Recombination

2.4.1. The Study of Jacob and Wollman

Jacob and Wollman (1955) studied the effect of UV on recombination of λ phage. In a two-factor genetic cross between phage having markers normally showing a 6% recombinant frequency, a UV dose of 67 J/m^2 delivered to both parental phage either before infection or soon after infection raised the recombinant frequency to about 30%. (Irradiation of one parental phage type gave only an increase to 15%.) Unlike the results of Kellenberg and Weigle (1958), irradiation of the host bacteria (*E. coli* K12S) prior to infection with the parental phage, irradiated or not, resulted in no increase over the result obtained when the host was not irradiated. The reason for the difference may have been due to the greater doses used by Kellenberger and Weigle, although other factors have certainly not been excluded.

When measured as a function of UV dose to biparentally infected complexes, recombination between closely separated markers increased (linearly) more slowly with dose than did that between more distantly separated markers (Fig. 3), the slope reflecting the distance between markers only in a qualitative way. A plateau value of recombinant frequency was attained at a dose (relatively independent of the distance between the markers studied) of about 60 J/m^2. The plateau value of recombinant frequency was lower for closely spaced markers: markers

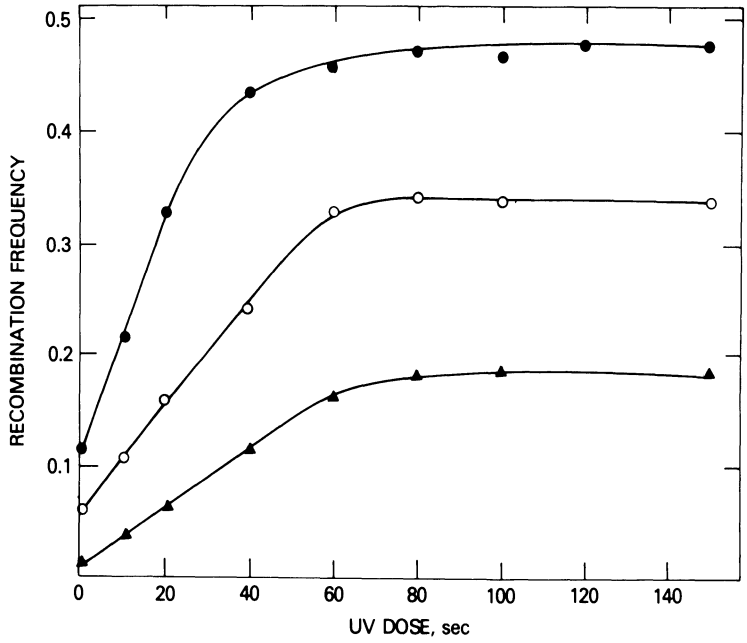


Fig. 3. Recombination frequency of λ phage irradiated after adsorption to host cells as a function of UV dose. Cells were infected in the presence of KCN at an m.o.i. of 10–15 with two phage strains differing by two genetic markers. After 20 min of adsorption, samples were diluted in cold buffer and irradiated. After 2 hr of growth at 37°C, free phage were plated with indicator bacteria. Dose rate approximately 0.8 J/m²/sec.

Symbol	Markers in cross	Recombinant frequency without UV
▲	$Ce_1 \times c$	1.5%
○	$g_1 \times c$	6.0%
●	$m_5 \times p_4$	12.0%

Redrawn from Jacob and Wollman (1955) with permission.

recombining at frequencies of 1.5%, 6%, and 12% with no irradiation recombined at plateau values 18%, 34%, and 48%, respectively, if the infected complexes had received at least 60 J/m². When infected complexes were incubated for timed periods before receiving 63 J/m² of UV, the induced recombination frequency dropped from the plateau level with a half time of 7–8 min postadsorption incubation to the value obtained with no irradiation when complexes were irradiated at 30 min of postadsorption incubation prior to receiving UV. (KCN was used during adsorption to synchronize infection.) Thus the ability of the infecting phages to have their recombination frequency increased by

UV light had a half-life of about 7 min. Similarly, if bacteria infected with two genetically marked irradiated phage populations (m.o.i. of 10–12 each) were subjected to photoreactivating conditions at zero time after KCN removal, the UV enhancement of recombination was not observed. If the complexes were incubated before receiving such photoreactivation treatment, the half-life of photoreactivability was also about 7 min, showing that the extent of the process which requires UV alterations to produce increased recombination frequency is 50% complete in 7 min.

The extent of recombination between two genetically marked phages in infected complexes which received no irradiation reached half-maximum value in about 25 min of postadsorption incubation, whereas that measured in irradiated complexes was already at maximum value at 25 min, the earliest time at which there were enough mature phage liberated to measure recombination frequency.

When a three-factor cross between two populations of genetically marked phage was arranged, and UV was delivered to infected complexes in increasing doses, the frequency of double recombinants was found both to be greater than that expected from the product of the two single recombinant frequencies and to be a linear function of UV dose (as was the frequency of the products due to either of the possible single recombination events). Thus the UV-enhanced double recombinants were suggested to arise from a single event rather than from two independent events. In this latter case, the frequency of double recombinants was expected to be proportional to the square of the dose. It certainly could be, however, that the expression of biological effects due to a single UV photoproduct requires a two-event response to the photoproduct.

If only one of the two phages were irradiated prior to its participation in a three-factor cross with one other phage, the majority of the double recombinants contained the middle marker of the irradiated phage and the outside markers of the nonirradiated phage, and single bursts that contained double recombinants usually contained only one of the two possible double recombinant products.

Thus Jacob and Wollman's UV-enhanced recombination (1) was earlier than general recombination, giving the half-maximum number of recombinants as early as 7 min postinfection, (2) was due to UV alterations that were reversed by photoreactivation, (3) increased as the distances between markers increased, and (4) gave rise to a higher frequency of double recombinants than expected, and did so in a nonreciprocal way.

2.4.2. Effects of a *uvrA* Mutation

Baker and Haynes (1967), following the results of Jacob and Wollman (1955), irradiated mixtures of λ*B*1 and λ*C*9 mutants, infected either AB1157 (*uvr*$^+$ *rec*$^+$) or AB1886 (*uvrA*, *rec*$^+$), and measured the recombinant frequency among the progeny. When infecting AB1157, the frequency of recombination between the phage measured at zero dose was elevated linearly in proportion to the UV dose received by the phage from 3% until a plateau was reached at a recombination frequency of 23% at a dose of 84 J/m^2. This result generally agreed with those of Jacob and Wollman (1955). In the *uvrA* AB1886 strain, however, the recombinant frequency initially increased about 4 times as rapidly as with AB1157 with UV dose to the phage, from 3% at zero dose to about 18% at 14 J/m^2 (about ten pyrimidine dimers per phage genome). Beyond this dose, the recombination frequency increased at the rate observed in the wildtype, but did not plateau, so that recombination reached 43% at about 125 J/m^2. Thus one would guess that UV-produced lesions in the phage (presumably including pyrimidine dimers) which were not repaired by AB1886 (and possibly 75% repaired in AB1157) caused the increase in recombination frequency. Clearly, because the conditions of the experiment required recombination, multiplicity reactivation of the phage was occurring. Since λ*red*$^+$ phage and *rec*$^+$ cells were used, both recombination systems would be expected to play a part in the UV-enhanced recombination. It would be of interest to dissect this phenomenon further using combinations of *E. coli rec*$^-$ and λ *red*$^-$ mutants as Radman *et al.* (1970) did earlier while studying host-cell reactivation. Recombinants generated by the *recA* system are reciprocal; those generated by the *red* system are not (Sarthy and Meselson, 1976).

2.4.3. DNA Synthesis

Cordone *et al.* (1975) confirmed the results of Baker and Haynes (1967) and showed functional λ*O* gene product (involved in λ DNA synthesis) to be required to observe an actual increase in the number of recombinants produced by UV irradiation of the two parental λ phage populations.

At 40°C, a cross between two *ts* λ*O*$^-$ mutants gave a twenty- to thirtyfold drop in the number of viable *N*$^+$-*R*$^+$ recombinants with respect to the number of recombinants from the same cross at 30°C (or

to a λO^+ cross at 40°C), but showed that products of a cross done under conditions of depressed DNA replication could be detected. Although the number of N^+-R^+ recombinants from a cross at 40°C of λO^- phages was not increased by low-dose UV irradiation of these λ parental phage prior to the cross (as it was sixfold in the case of λO^+ parents), the recombinant frequency increased with UV dose as if recombination itself favored the survival of UV-damaged phages under conditions of inhibition of DNA synthesis.

Another way of measuring UV effects on the recombination of λ phages under conditions of minimal transcription, translation, and DNA synthesis is to cross an infecting phage with the phage DNA carried by a homoimume lysogen and to measure the production of recombinants following subsequent induction. Such an approach was used by Lin and Howard-Flanders (1976). They infected *E. coli* (λ*cI857 P80*) strains with UV-irradiated λ*cI857 P3* and compared the UV-induced recombination with that obtained in similar (productive) heteroimmune crosses [*E. coli supE44* (λ*cI857 P3*) infected by λ*imm434 P80*]. In the homoimmune cross, 60 J/m² increased the recombinant frequency from 0.05% to a maximum 0.4% in both *uvrA* and uvr^+ lysogens. In the heteroimmune cross, however, the recombinant frequency greatly increased with dose to about 4% in the *uvrA* strain at 100 J/m² to the phage and to 1% in the uvr^+ strain with phage that received 150 J/m². Such an increase did not occur in a *recA* strain. Thus, in the absence of vegetative phage biochemistry (homoimmune cross), the UV-induced increase in recombination was low and independent of *uvrA* function. A phage treatment known to result in the production almost exclusively of pyrimidine dimers (acetophenone-produced DNA photosensitization to 313 nm light) produced almost no increase in recombination frequency in the homoimmune cross. It was stated by the authors, therefore, that most of the observed increase in recombinant frequency was probably due to nondimer DNA photoproducts. In the heteroimmune cross, on the other hand, similar studies, together with studies using photoreactivation, implicated dimers as contributing greatly to the recombination stimulated by UV irradiation of the phage.

Blanco and Armengod (1976) did a slightly different experiment. They infected various *434hy* lysogens with λ*cI857 O am29*. Recombinants having λ immunity and O^+ function were assayed by infecting su^- *434hy* lysogens. Because intact λ*O* gene is required for λ DNA synthesis, the recombination experiment could be studied in the presence or absence of DNA synthesis by infecting su^+ or su^- hosts. By appropriate crosses, it was found (under conditions permitting DNA

synthesis) that *red* function (acting in *recA* hosts) promoted about fifteenfold more recombination (recombinants per infected cell) of non-UV-treated phages than did *recA* function acting on *red*$^-$ phages. But when the phage were UV treated, *red* function in the absence of *recA* product had little effect on the number of recombinants produced per infected cell. However, a UV-dose-dependent fiftyfold increase in the recombination frequency of *red*$^-$ phages was observed in the presence of *recA* product, thereby giving a five- to tenfold higher recombination frequency than that observed in *recA* hosts infected with *red*$^+$. By contrast, under su$^-$ conditions where phage DNA synthesis was not permitted, *red* promoted (in a *rec*$^-$ strain) recombination was about fiftyfold less efficient than when DNA synthesis was allowed to occur. The level of *rec* mediated recombination of *red*$^-$ phages was nearly independent of whether or not DNA synthesis took place.

Kobayashi and Ikeda (1977, 1978) studied *recA*-dependent λ recombination in the presumed absence of phage metabolism by crossing two irradiated λ *red* mutants in *recA* and *rec*$^+$ hosts in the presence of chlorampenicol and rifampicin, and extracting, then packaging the resultant DNA *in vitro*. The observed recombination, requiring about 1 hr for completion, was about 300-fold more efficient in rec$^+$, *recB*, or *recC* hosts than if the hosts carried *recA* or *recA recB*. This would seem to be a useful system to employing for dissecting UV-enhanced recombination phenomena.

2.4.4. Effects of Gyrase Inhibitors

Hays and Boehmer (1978) used a novel recombination assay to study the effects of gyrase inhibitors on UV-induced λ recombination. λ*a106-19 b538* is a λ phage both deleted in 16% of its genome length and carrying an 18% of λ length tandem duplication of genes *A-V*. Because of its overly long genome, it was sensitive to killing by EDTA, where as an intra- (or inter-) molecular recombinant, λ*b538*, having lost the duplication (*a106-19*), was not. Kinetics of both recombination and repair of λ DNA were measured by using EDTA to select for recombinant phage made in a lytic spheroplast transfection assay of phage DNAs prepared from samples taken at timed intervals during infection. To measure effects due only to cellular *recA* function during the primary infection, λ phage strains mutant at *red* were used, and *recA* cells were selected as hosts in the transfection assay of the λ DNAs. A recombinant level of 10% (in 150 min of postinfection incubation) was

measured when nonirradiated phages infected a *rec*$^+$ (but not a *recA*) primary host capable of supporting lytic phage development. Nonirradiated phage infecting homoimmune lysogens recombined at low levels, but, in a similar infection, the DNA from phage irradiated with 190 J/m^2 of UV was found to be about 55% recombinant after 120 min of incubation. Further, *recA*-dependent repair was observed as a tenfold increased infectivity of the DNA prepared from samples during the infection. Both the observed repair and recombination were strongly inhibited if the primary infection took place in the presence of coumeromycin or oxolinic acid, inhibitors of *E. coli* gyrase.

2.4.5. Formation of Joint Molecules

Takahashi (1977) measured the production of joint (recombinant) DNA molecules (by CsCl banding and electron microscopy) during an infection of various *E. coli recA recB* strains by irradiated mixtures of light and BrdUrd heavy λ*cI*857 O^-P^- phages (blocked in DNA synthesis). A UV dose permitting 1–3% phage survival increased from 2–3% to 10–12% the recovery of joint molecules extracted 30 or 50 min after infection. Evidently *red*-mediated recombination was responsible for the increase, although such a dependence was not demonstrated by the use of phage mutant at *red*. Using host bacteria that also harbored a temperature-sensitive *polA* mutation (*polA recA* is a lethal combination), such joint molecules were found at about 10% frequency. When the phages were irradiated and used for infection, joint molecule formation increased to about 40%, suggesting that unfilled gaps can promote recombination. These results must be interpreted with some caution, however, since irradiation of DNA that contains BrdUrd results in the production of DNA photoproducts different from dimers.

2.4.6. Summary

The picture of the mechanism of UV-enhanced recombination is far from clear. The following generalities can be made. *RecA*-dependent recombination among two infecting λ *red*$^-$ mutants was suppressed in homoimmune lysogens. The same is true for *recA*-dependent recombination between one infecting phage and the resident homoimmune prophage (Lin and Howard-Flanders, 1976). If the phage were irradiated in the former case, *recA* dependent recombination occurred to a great extent, but in the latter case to only a very limited extent. It

would be interesting to determine the reason for this discrepancy. When infecting lytically, *recA*-dependent UV-enhanced recombination (assayed by biological activity) occurred in a *red* independent *uvrA* dependent fashion (Baker and Haynes, 1967; Lin and Howard-Flanders, 1976; Hays and Boehmer, 1978). The apparently anomalous observation that joint molecule production is enhanced by irradiating λ *red*$^+$ phage prior to infection of *recA* hosts (Takahashi, 1977) may be due to the fact that half of the phage population contained BrdUrd as a partial replacement for TdR in its DNA.

The mechanisms leading to the observations called multiplicity reactivation and UV-enhanced recombination are presumably closely related or identical. Whether multiplicity reactivation is due to the preferential survival of phage among which increased recombination has occurred, or whether UV-enhanced recombination is due to some repair mechanism that produces potential recombination points at sites of photochemically altered DNA bases, thereby leading to increased recombination, cannot be sifted out from the present data.

2.5. Weigle Reactivation (WR) and Weigle Mutagenesis (WM)

Weigle (1953) found that a relatively small UV dose delivered to the λ host, *E. coli* K12S, prior to (or early after) infection increased the survival of the irradiated λ phage without altering the number of plaques due to unirradiated λ phage (Fig. 4). The effect was maximal at a dose to the bacteria of about 60 J/m^2. The magnitude of the restorative effect was estimated by noting that the UV survival curve of λ phage on *E. coli* K12S (irradiated to produce the maximum increase in phage survival) had a slope of about half of that obtained if the bacteria were not irradiated. Conceptually, then, it appeared that half of the lethal UV damage in the λ phage was reversed by UV pretreatment of the host bacteria. Weigle found a large increase in the number of clear plaque mutants among the phage survivors, ranging to 2.5% of the total number of plaque formers, but only if both the phage and bacteria had received optimal UV doses (450 J/m^2 to the phage, 60 J/m^2 to the bacteria).

Thus WR of phage is said to occur when the survival of UV-irradiated λ phage is increased by preinfection UV irradiation of the host. Such reactivation is generally accompanied by elevated mutagenesis of the reactivated phage. In a typical case, increasing the UV dose to the bacteria and measuring the plaquing ability of phage that have received a single dose of UV, the survival of phage first increases,

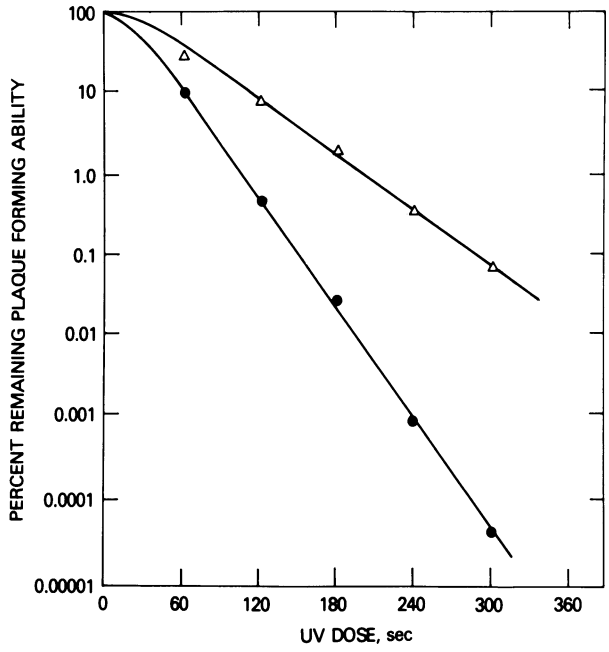


Fig. 4. Survival of λ phage as a function of UV dose. Dose rate was about 1.5 J/m²/sec. Survival was measured using nonirradiated bacteria (●) or bacteria that had received 60 sec of UV (Δ, a dose found to give maximal WR). Redrawn from Weigle (1953).

attains a maximum, then actually decreases with greater UV dose to the host due to loss of cellular capacity to support phage growth (Fig. 5; Kellenberger and Weigle, 1958). The phenomenon occurs using either λ^+ or λ vir, which ruled out the hypothesis that WR is simply due to an induction of lysogenized UV-irradiated phages. WM is defined as the mutagenesis that accompanies WR.

2.5.1. The S.O.S. Hypothesis of Radman

Radman (1974), sensing a common biochemical basis for the phenomenological similarity between the induction of λ and the phenomena of WR, WM, bacterial mutagenesis, filamentation, and various apparently related observations, proposed the S.O.S. hypothesis. According to the hypothesis, the above phenomena were supposed to be due to the action of an error-prone repair process the components of which were synthesized after a metabolic derepression in response to UV damage. It is a very simple, constructive, unifying

hypothesis on which much experimentation has been based, and is generally regarded as a breakthrough in the field. However, as with all breakthrough ideas, it is difficult to recapture the complexity of hypothetical biochemical constructs that seemed to be required to account for the data as they existed prior to the hypothesis.

Data relevant to phage induction, Weigle reactivation, and Weigle mutagenesis are presented in this chapter in the absence of a stated S.O.S. framework in an attempt to look closely into nooks and crannies not necessarily related either to S.O.S. correlates or to proof or disproof of the hypothesis. Nevertheless, much of the presentation of these sections is obviously molded on the template of Radman's idea.

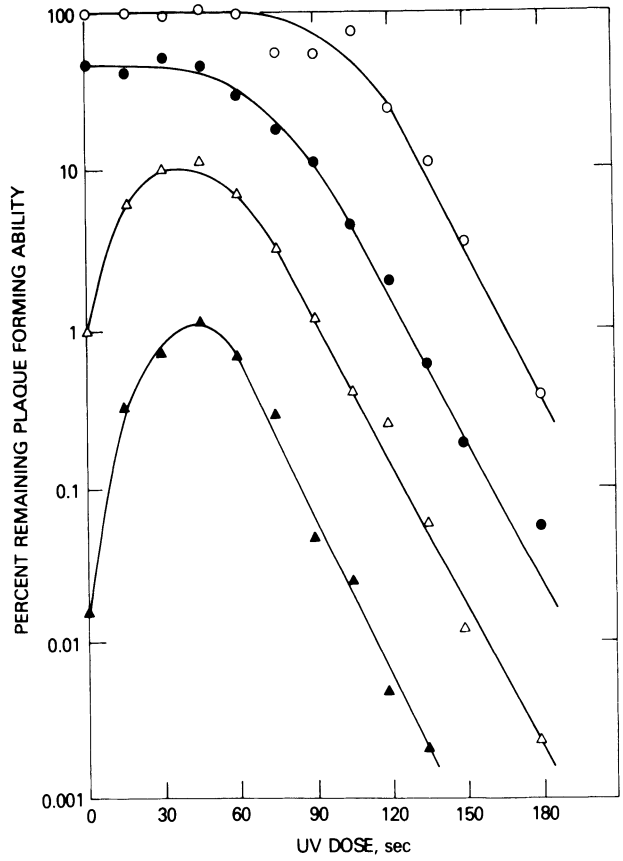


Fig. 5. Plaque formation by unirradiated and irradiated λ phage as a function of UV dose delivered to the host (*E. coli* K12S). The infecting phage received no UV (○), a dose leaving 50% survival (●), a dose leaving 1% surival (△), and a dose leaving 0.01% survival (▲), when nonirradiated bacteria were used. Redrawn from Kellenberger and Weigle (1958).

2.5.2. Dependence of WR on *uvr* Functions

Harm (1963*a*) showed UV-irradiated λ vir *b*2 to be subject to Weigle reactivation when infecting either UV-irradiated *E. coli* K12S, *E. coli* C, or their *uvr*$^-$ mutants *E. coli* K12s *hcr*$^-$ (*uvrA*) and *E. coli* C *syn*$^-$ (*uvrC*). (See Fig. 6.) In the cells with wild-type repair, somewhat more WR occurred than in the *uvr*$^-$ strains. The dose to the *uvr*$^+$ bacteria giving maximum WR was 54 J/m^2 and that in the *uvr*$^-$ cells was 6 J/m^2 (K12*hcr*$^-$) or 10 J/m^2 (*Csyn*$^-$).

Mattern *et al.* (1965), on the other hand, found a somewhat different result. Their *uvrA* strain (KMBL99 *dar3*) irradiated with 30 J/m^2 (the lowest dose used) gave an amount of WR of λ equal to that afforded by their *uvr*$^+$ derivative (KMBL49) irradiated with 60 J/m^2 where the effect was maximum. However, a mutant mapping near *uvrC* (KMBL92 *dar4*) gave WR at 6-10 J/m^2, which became negative at 30

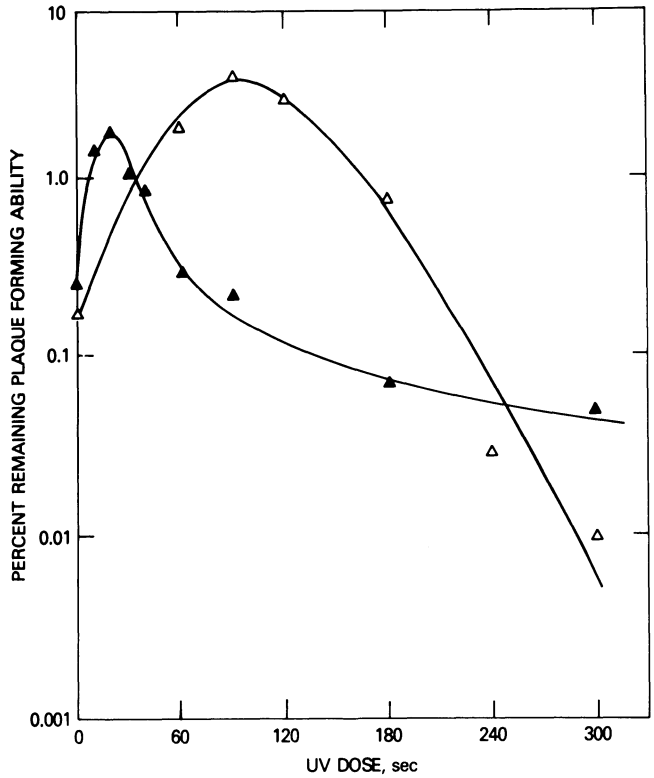


Fig. 6. W reactivation of UV-irradiated λ phage as a function of UV dose to the bactieral hosts. △, *E. coli* C (UV dose to λ was 360 sec); ▲, *E. coli* C *syn*$^-$ (UV dose to λ was 50 sec). Dose rate was about 0.6 J/m^2/sec. Redrawn from Harm (1963*a*) with permission.

J/m^2. Two *uvrB* mutants (KMBL90 *dar1* and KMBL101 *dar6*) gave only negative WR (i.e., the survival of the UV'd λ is less than if non-irradiated cells are infected), but the lowest dose that these *uvr*$^-$ derivatives received was 30 J/m^2, three- to fivefold greater than the dose reported by Harm (1963*a*) to give maximum WR in *uvr*$^-$ hosts, and probably overshot the dose giving maximum induction by that factor.

Ogawa *et al.* (1968) showed WR of UV-irradiated λ phage to occur nearly normally in a *uvrD* strain, very poorly in a *uvrC* strain, and not at all in a *uvrA* or a *uvrB* strain. Thus various *uvr*$^-$ mutants mapping in the same region appeared to give different results in a WR experiment. This may be due to the nature of the product produced, i.e., whether it is due to a gene with a point mutation, deletion, or termination. Defais *et al.* (1971) suggested that the difference is due to the fact that UV doses applied to the phage and to the host were different. This is only partly true.

In any case, Hart and Ellison (1970) reported WR to occur in Harm's *E. coli* K12 *hcr*$^-$ (*uvrA*) and in the *E. coli* K12 mutants (obtained from Howard-Flanders), AB2434 (*urvB*), and AB2433 (*uvrC*). Kneser (1968) also repeated the experiment using Harm's K12S *hcr*$^-$, and Defais *et al.* (1971) found WR in the AB1886 *uvrA* mutant, while Radman and Devoret (1971) found WR in AB1886 (*uvrA*), GY471 (*uvrB*), and KMBL100 (*uvrC*). In all cases, the doses needed to achieve maximal WR in *uvrA* and *uvrB* strains were between 5.0 and 10 J/m^2, 5 and 25 J/m^2 in *uvrC* strains, and, in the *uvr*$^+$ strains, between 30 and 60 J/m^2. Thus, in strains that do show WR of UV-irradiated λ, the effect is reproducible from laboratory to laboratory, and WR appears to occur in strains carrying mutations in all of the four *uvr* loci discussed.

A priori, the interpretation of the dose-dependent difference in WR observed in *uvr*$^+$ and *uvr*$^-$ strains is not straightforward. It is tempting to hypothesize that, after cellular DNA synthesis in the UV-treated bacterium, any remaining unrepaired lesions cause the induction of an enzymatic repair process. Thus, in cells capable of repair, more UV would have to be delivered to induce the repair process. This widely accepted interpretation may be true. It should be noted, however, that the experimental design of most experiments of this nature entails employing two populations of irradiated λ. One population is relatively lightly irradiated (36 J/m^2, giving about 23 dimers/genome) to about 0.1% survival, measured using the uvr$^-$ strain; the other population is irradiated with about sixfold more UV to obtain the same survival when infecting the *uvr*$^+$ strain. On infection of nonirradiated *uvr*$^-$ bac-

teria, little of the damage in the DNA of lightly irradiated λ phage is removed. In contrast, the majority of the damage (presumably mainly pryimidine dimers) in the DNA of the more heavily irradiated λ is removed by excision on infection of the *uvr*$^+$ bacteria. Thus one way to view the induced repair that occurs in a WR experiment is to view it as repair that occurs over and above any excision repair that the DNA of the phage may incur on infection. During WR of the lightly irradiated λ by the *uvr*$^-$ bacteria, most of the phage DNA damage presented to the WR repair system is pyrimidine dimers. However, in the case of the *uvr*$^+$ bacteria, which efficiently remove pyrimidine dimers from the phage DNA, most of the DNA damage to be repaired by WR would likely be nondimer damage. Thus an alternative hypothesis for the difference in WR dose dependence observed between *uvr*$^+$ and *uvr*$^-$ bacteria would be that low UV doses to the bacteria induce repair that reverses the biological effects due to pyrimidine dimers, while larger doses are required to induce repair that reverses effects due to nondimer damage.

2.5.3. Dependence of WR on the Other Repair Functions

2.5.3a. Polymerase I

Caillet-Fauquet and Defais (1972) established that the *polA* mutant JG112 could perform WR of UV-treated λ phage most efficiently if irradiated with 7 J/m^2 of UV. The JG113 *polA*$^+$ strain required 20 J/m^2 to perform maximal WR. Breseler *et al.* (1978) confirmed such *polA* dose dependence of WR. Thus *polA* strains behave quite similarly to *uvr*$^-$ strains in requiring relatively low WR induction doses.

2.5.3b. *Rec* and *lex* Functions

Kneser (1968) showed that a *rec*$^-$ strain (*rec*$^-$*152* of M. Meselson) gave only negative WR. Ogawa *et al.* (1968) obtained very similar results using their W3623 *recA42* strain. Hart and Ellison (1970), extending the phenomenon to a *recC* strain, showed the negative WR in KMBL *recC38* and KMBL *recA36* to be experimentally indistinguishable. Defais *et al.* (1971) found that, in addition to AB2463 (*recA13*), neither the AB2494 (*lexA*) nor AB2472 (*uvrA6 lexA*) derivatives showed any detectable WR. Radman and Devoret (1971) showed that,

while the *recA* function of *E. coli* was necessary for successful WR, the λ *red* function was not. Moreover, λ*b2* (lacking about half of the phage attachment region) underwent WR just as λ^+ did in a *uvrB* host either deleted or not in the λ*att* region. These last findings give little support to models requiring recombination between host and bacterial DNAs to account for the requirement for the *recA* gene product during WR.

Kerr and Hart (1972) showed WR of λ*vir* to occur in both AB2470 (*recB21*) and in JC 5489 (*recC22*) but in neither the *exrA* (*lex*) strain PAM5717 nor the *recA* JC5547 strain. λ*int102 red3* underwent successful WR with the same kinetics as λ*vir* in the *recB21* host, showing these two λ functions not to be required in the process.

Breseler *et al.* (1978) found, in addition to positive WR in the wild type and in a strain carrying *uvrE502*, no or negative WR to be observed in a temperature-sensitive ligase mutant 2668 *ligts7* at 37°C, in a *lexA102* strain, in *recA13*, and in a *recA13 recB21 recC22* strain. The induction of WR in *recL* mutants showed the same dose response as did a *urvB* mutant with a maximum at 10 J/m^2 (Rothman *et al.*, 1979). In the same study, *recF* mutants showed reduced WR, and no WR was observed in strains mutant at both *uvrA* (or *uvrB*) and *recF* (or *recL*).

Thus, of the *rec* mutants studied, only *recA* gene function appears to be absolutely required for successful WR, while the *recBC* nuclease does not appear to be necessary. Further, *lex* (*exr*) function is also needed. Mount (1977) has investigated closely the role of *lex* function in WR by comparing his two strains (described earlier in the section UV induction of λ) DM1180 and DM1187. DM1187 has a *spr* mutation mapping at *lexA* in addition to the *lexA tif1 sfiA* mutations present in DM1180 and produces constitutive levels of protein X. A plausible explanation for this effect is that the *spr* mutation results in inactive *lexA* product, the presumed repressor of the *recA* operon. If this were so, then nonirradiated DM1187 would behave in WR experiments as if it had been UV irradiated. Indeed, this proved to be true: the inactivation slope of UV-irradiated λ phage using nonirradiated DM1187 was half that measured when using its nonirradiated parent DM1180 as a viral host, showing DM1187 to have constitutive WR.

2.5.4. Induction and WR May Be Due to Different Mechanisms

Kato and Shinoura (1977) isolated three classes of *E. coli umu* mutants (in which UV caused no mutagenesis). Markers from two such classes mapped at *recA* and *lexA*, but the third, *umuC*, mapped

between *purB* and *hemA*. Three different mutant *umuC* alleles were transduced into a *uvrA* strain. λ was UV induced in these strains just as well as in the *urvA* parent, but no WR was observed.

2.5.5. Dependence of WM on Cellular Repair Functions

Among the λ phage that undergo WR are found a high proportion of mutants apparently arising from a biochemical process very similar to that which leads to WR. By far the most common mutation studied is that originally studied by Weigle (1953), the production of clear plaque mutants, λ*c*, from UV-irradiated λc^+ strains. (Thus WM of λ*vir* or λc^- mutants is not usually studied.)

Miura and Tomizawa (1968) found that, even though they observed little WR in the N17-9 *uvrA54* mutant (as noted previously, Ogawa *et al.*, 1968), Weigle mutagenesis did occur. The frequency of λ*c* mutants among λ phages that received 44 J/m^2 increased from about .09 to about 1.2% as the dose to the bacteria increased from 0 to about 6 J/m^2 of UV. In the *uvr*$^+$ W3623 strain, WM occurred to a similar extent, but the dose giving maximal WM was much higher, about 55 J/m^2. Using a single test dose to the bacteria, the authors found no WM to occur in any of three *recA* mutants, but found some in a *uvrB* and in a *uvrD* strain. In the same vein, Defais *et al.* (1971), using AB1886 (*uvrA*), confirmed in general the behavior for the *uvrA* mutant reported above, but showed that while 7 J/m^2 of UV to AB1886 induced maximal clear plaque mutant production (0.25%) in λ populations irradiated with 20 J/m^2, only 3 J/m^2 (the lowest dose used) induced the maximal clear plaque mutant production (0.37%) in λ irradiated with 50 J/m^2. They also showed deficient WM by AB2463 (*recA*) and AB2494 (*lexA*). Breseler *et al.* (1978) used another mutation assay to measure WM using to advantage the fact that λ11C (λcIv_2v_3) forms no plaques when plated on a lysogen, but forms clear plaques on acquisition of the v_1 mutation which converts it to λ*vir*. They found that all except one of those strains which showed WR showed WM. Wild-type (AB1157) bacteria, a *polA* strain, and a *recB recC sbcB* strain all showed positive WM. However, a strain carrying the *uvrE*502 mutations, while showing WR, showed no WM. (Because *uvrD* and *uvrE* have been reported to define the same gene (Kushner *et al.*, 1978), the observation that a *uvrD* mutant showed WR (Miura and Tomizawa, 1968), but that a *uvrE* mutant did not, would appear to be anomalous and deserves further work.) The rest of the strains (*recA13*, *lexA102*, and *ligts7* at 37°C) showed little or no WM.

The cumulative evidence shows that many genes play a role in WM and that those are the same genes that control WR. The *recA*, *lexA*, *uvrE* (*recL*), and *lig* genes may be required absolutely, with the *uvr* genes modulating the expression of both phenomena.

2.5.6. Indirect WR and WM

Like the induction of λ, WR and WM can also be provoked by the intracellular introduction of UV-irradiated DNA. Thus George *et al.* (1974) and Miura (1974), using irradiated *Hfr* and λ-resistant F^+ donors, induced both WR and WM of λ phage in λ-sensitive recipients. Miura (1974) established that, as for direct WR and WM, *recA* function is required for indirect WR and WM. The finding that WR and WM occur in response to DNA transferred from an irradiated *Hfr* is quite interesting, since, as discussed earlier, an irradiated Hfr does not produce indirect induction of λ. George *et al.* (1974) pointed out their intriguing anomalous observation of reduced direct WR of irradiated λ (grown prior to irradiation in a strain lysogenic for P1) when infecting an irradiated bacterium not lysogenic for P1, perhaps suggesting a role for DNA methylation in WR. This is not unreasonable since strains carrying the *dam* mutation and, thereby, having reduced amounts of 6-methyladenine in their DNA, are nonviable when they also carry *recA*, *polA*, *recB*, or *recC* mutations (Marinus and Morris, 1974). Further, Day (1977), following the original finding of Bertani and Weigle (1953) that UV-irradiated *E. coli* lost their ability to restrict unirradiated λ phage grown in *E. coli* C, found such UV-produced loss of restriction to occur at lower UV doses in a *uvrA* mutant. No such loss was observed either in a *lexA* or in a *recA* mutant. Based on this finding, UV would be expected either to induce an increased methylase activity in *E. coli* or to result in the inhibition of the K-specific restriction enzymes themselves.

2.5.7. Kinetics of WR/WM Expression and Inhibition

Defais *et al.* (1976) showed that a 37°C postirradiation incubation of AB1886 (*uvrA*) caused parallel increases in both WR and WM of λ in a time-dependent manner. Both W phenomena become maximal at about 25 min of incubation and decreased with a half-life of about 1 hr thereafter. The presence of chloramphenicol with the irradiated bacteria

until the time of infection by irradiated λ severely decreased the observed WR and WM.

Caillet-Fauquet and Defais (1977) found that incubation of a (ts) *dnaB* mutant (GY953) at 42°C produced a parallel induction of both WR and WM of irradiated λ (infection and plaque formation was carried out at 32°C). The maximal effect occurred after about 90 min of incubation at 42°C. A temperature-independent revertant showed no such induction of either phenomenon. Castellazzi *et al.* (1972) studied the pleiotropic properties of their thermosensitive (*recA*) mutant *E. coli* C600T44 called *tif* (described previously). They found that a 42°C treatment JM12 *tif-1* (*tif-1* transduced from C600T44 to strain JM-1 by P1) produced both greater survival and greater mutagenesis of irradiated λ than the same treatment given to JM-1. Such induction appeared maximally at 40 min.

The kinetics of induction of the intracellular capacity to perform WR and WM are not reproducible from system to system. However, in all three cases discussed, both phenomena were induced concomitantly, and the data are consistent with the notion that WR and WM are due to the same molecular process.

2.5.8. WM without UV Irradiation of λ

Ichikawa-Ryo and Kondo (1975) performed an important experiment showing that UV irradiation of *E. coli* produces a mutagenic environment even for an unirradiated infecting λ phage. (Although previously a slight increase in mutation frequency of unirradiated λ over that occurring in unirradiated λ hosts had been observed when infecting UV-irradiated hosts, a high level of quantitation of this difference was difficult because such mutagenesis was quite infrequent.) The authors used an indicator strain NO483 which lysogenizes λc^+ almost 100% of the time, giving plaques only when infected with λc^-, and thereby were able to measure λc^- without looking through a great number of λc^+ turbid plaques. Interestingly, even without UV damage in the DNA of the infecting λ phage, UV irradiating the host produced significant increases in λc^- yield in the now familiar way: The AB1886 *uvrA* mutant required less UV than the uvr^+ W3623 strain to perform such WM, AB2463 (*recA*) showed the lowest amount of WR, with a *lex* mutant (DM844) showing a reduced but significant level. Thus the UV-induced mutagenic environment of the wild-type *E. coli* is dependent on the DNA repair capacity of the cell. While such an environment is highly conducive to the mutagenic repair leading to survival of UV-

irradiated (WR), UV damage in the phage DNA is not necessary for that environment to be mutagenic.

2.5.9. WM without UV Irradiation of *E. coli*—The Role of *lexA*

At 42°C, the *tif* mutant performs WM without being irradiated (Castellazzi *et al.*, 1972). While investigating the biological role of *lexA* using *lexA* in a *tif-1 sfi* background, Mount (1977) characterized DM1187, a *spr* mutant of DM1180 (*spr* maps at *lexA*) in which both constitutive protein X production and constitutive WR occur, as described previously. When infected with UV-irradiated λ, DM1187 (but not DM1180) gave rise to a high level of clear plaque phage mutants among the progeny, behaving as if it had been UV irradiated. Thus both WM and WR can occur in *E. coli* containing a mutation in the *lexA* gene if the *E. coli* is also mutant at *spr*. The gene represented by *lexA* thus appears to play a role in regulating WR/WM, as opposed to being a structural gene whose product is directly involved in these processes.

2.5.10. WR Assayed Using Infectious λ DNA

Wackernagel and Winkler (1971) found spheroplasts to be incapable of WR and WM. They isolated a mutant (*mul*, mapping between *pyrE* and *ilv*) which gave rise to greatly increased mutagenesis of UV-irradiated λ phage. The authors found that λ irradiated with 320 J/m^2 survived equally well (0.04%) in AB1157 or in WA10, a *mul* mutant, but, in log-phase nonirradiated cells, λ*c* mutants were found with a frequency of 5×10^{-4} in AB1157 and in WA10 about 4×10^{-3}. When infecting stationary phage bacteria, λ survival was still the same using both strains at 320 J/m^2 (0.1%), but the mutation frequency increased to 3×10^{-3} AB1157 and to 9×10^{-3} in WA10. However, with increasing doses of UV treatment to the spheroplasts used to measure the survival and mutagenesis of UV-treated λ DNA, neither WR nor WM was detected.

Later, however, using AB1157 to compare the behavior of the spheroplast assay with that of calcium-treated cell transfection system, Wackernagel (1974) found the use of the latter to give rise to both WR and WM of infectious λ DNA. It is conceivable that, during the preparation of the spheroplasts, some *rec-lex*-related protein (protein X?) is lost from the cell. That the survival curve of UV-treated λ DNA

measured by the spheroplast assay is about 20% steeper than that obtained with the calcium system is consistent with this interpretation.

A similar approach was taken by Tomilin and Mosevitskaya (1973, 1975). These workers showed that, while the background production of clear plaque mutations in nonirradiated λ was increased when using the calcium/DNA system, it was still possible to demonstrate both WR and WM of λ infectious DNA, and, further, that both W functions were dependent on *recA* function. In an experiment designed to test the effect of DNA incision on WR and WM, irradiated λ DNA was treated with an enzyme preparation from *M. luteus* which makes single-strand breaks in DNA containing pyrimidine dimers (a step that the authors show to circumvent the requirement for *uvrA*, *uvrB*, and *uvrC* function in the repair of UV-treated λ DNA). The calcium-treated host bacteria (either AB1157 or AB1886 *uvrA6*) were irradiated to induce WR/WM, and were infected with the irradiated DNA either treated or not with the enzyme. Compared to the results obtained without enzyme treatment, both WR and WM were found to be strongly depressed if the DNA had received the enzyme treatment.

2.5.11. Other Findings

Mount *et al.* (1976) did a biological experiment showing that under certain conditions WR *could* be separated from WM. A strain carrying *recA*1 and *tsl-1* (*tsl*, as discussed earlier, is a temperature-dependent UV-resistant revertant of *lex* mapping at *lex*) was constructed. It performed host-cell reactivation of UV-irradiated λ phage as well as the rec^+ tsl^+ strain but better than a *recA*1 tsl^+ strain, showing the *tsl* function could phenotypically reverse the *recA* host cell reactivation defect. When irradiated, the *recA*1 *tsl-1* strain showed a limited but positive WR of UV-irradiated λ phage which plateaued at low UV doses to the bacteria. However, no (or slightly negative) WM was detected. The authors proposed a UV-produced induction of an error-free repair pathway to account for their effect. In agreement with this finding, Breseler *et al.* (1978) showed WR, but no WM, in *uvrE502*, as noted previously.

Lytle *et al.* (1978) showed that the protease inhibitor, antipain, blocked WR of UV-damaged λ phage when infecting irradiated *E. coli*. Thus the action of a proteolytic enzyme is required for successful WR to occur, as it is for UV-produced mutagenesis, λ repressor inactivation, and filamentation (Meyn *et al.*, 1977).

In an attempt to correlate the possible involvement of closed circular DNA with WR, Ogawa and Tomizawa (1973) infected UV-irradiated W3623 (λ*cI ind*$^-$) with UV-irradiated λ*cI* and incubated with and without chloramphenicol (CM). After 75 min, the λ*cI* DNA was isolated and assayed for the percent of DNA found as closed circles. In the presence of CM (or when the irradiated λ was used to infect unirradiated cells without CM), 5% of the DNA was found as closed circles, whereas 25% of the DNA of the irradiated phage was found as closed circles without CM treatment. Because the survival of the UV-irradiated λ*cI* was 10% and 30% on unirradiated cells, respectively, it is tempting to postulate that WR reverses the inhibition (caused by UV-irradiating the phage) of conversion of linear λ DNA to λ closed circles that occurs in nonirradiated *E. coli*.

Thus the picture we get from the studies on WR and WM is not much unlike that discussed for the UV induction of λ phage. Following the material discussed, a well-chosen UV dose delivered to a wild-type host bacterium causes the chloramphenicol-sensitive production of a mutagenic intracellular environment, presumably due in part to the synthesis of proteolytic enzymes. Such an environment favors the survival of UV-irradiated λ phage. The *uvr* and *polA* functions of the host act to decrease the UV-produced WR/WM-inducing signal, presumably by repairing UV products which either are or, under appropriate conditions, lead to the signal. After infection, the induced system acts to aid circularization of damaged, linearly injected λ DNA, a process normally blocked by UV damage in the DNA. The wild-type *uvrE* (*recL*) product plays its mutagenic role in repair (or in DNA replication) on a repaired template and phage production goes on.

2.5.12. Addendum to WR/WM

It is not at all clear, of course, that the etiology of mutagenesis in bacteria or bacteriophage is related to that of carcinogenesis. However, the use of bacterial and/or phage mutation systems in carcinogen detection schemes has gained a certain popularity over the past few years. The phenomena, designated WR and WM here, are not found only when UV is used as the inducing agent. It is becoming apparent through the work of Devoret and collaborators (Sarasin *et al.*, 1977) that WR of UV-irradiated phage is observed if an appropriate *E. coli* host is treated with "ultimate" carcinogens or carcinogens activated by mixed-function oxidase preparations. The induction of λ has also been used as such a test system (Moreau *et al.*, 1976).

Whether or not human carcinogenesis can be accurately predicted on the basis of results obtained with bacteria, one benefit of establishing the molecular mechanism of WR/WM is that it can inspire molecular experiments designed to unravel problems of human carcinogenesis.

2.6. Prophage Reactivation

Jacob and Wollman (1953) observed that λ mutants (virulent, *v*) could be isolated which have the property of forming plaques on lawns of *E. coli* K12 lysogenic for λ phage (*E. coli* K12(λ)) as well as on *E. coli* K12S lawns, whereas wild-type λ formed plaques only on the latter. Thus these authors were able to obtain UV survival curves for λ*v* using both *E. coli* K12S and *E. coli* K12(λ) as hosts. It was found that λ*v* was about twofold more UV resistant when assayed using *E. coli* K12(λ) than when using *E. coli* K12S as hosts (Fig. 7). This phenomenon is now called prophage reactivation. When using λ*gv*, a double mutant having both virulent (*v*) and large-plaque (*g*) markers, in prophage reactivation experiments, a dose-dependent proportion of λ recombinants (up to 17%) was found if the λ*gv* received irradiation prior to infecting *E. coli* K12(λ^{++}), leading to the belief that prophage reactivation works through recombination. These workers also showed that λ*v* underwent WR when infecting either UV-irradiated *E. coli* K12(λ) or UV-irradiated *E. coli* K12S. Thus prophage reactivation did not eliminate the damage alleviated by Weigle reactivation.

Prophage reactivation is now thought to be due almost entirely to recombination between the infecting phage and the resident prophage, perhaps suggesting why the reactivation factor is only a factor of about two (i.e., prophage reactivation reduces the effective UV dose by a factor of 2).

Hart and Ellison (1970) and Kerr and Hart (1973) used λ*v*, as did Jacob and Wollman (1953), to study prophage reactivation in lysogens of *E. coli* C600. They used, as prophages, phages 424 and 434 (both of which recombine with λ, but only 434 integrates in the same (*gal/bio*) region in which integrated λ is found) and the noninducible prophages 18 and 186, which are known not to recombine with λ. Of these, only 434 gave prophage reactivation. The extent was equal to that afforded by λ^+ lysogens. The use of a double lysogen, C600 (λ, 434), gave rise to an almost additive effect, the inactivation slope of the UV-damaged λ being reduced about fourfold in the presence of the two prophages. This finding is consistent with the idea that doubling the number of possible recombination events doubles the reactivation. Using lysogens of

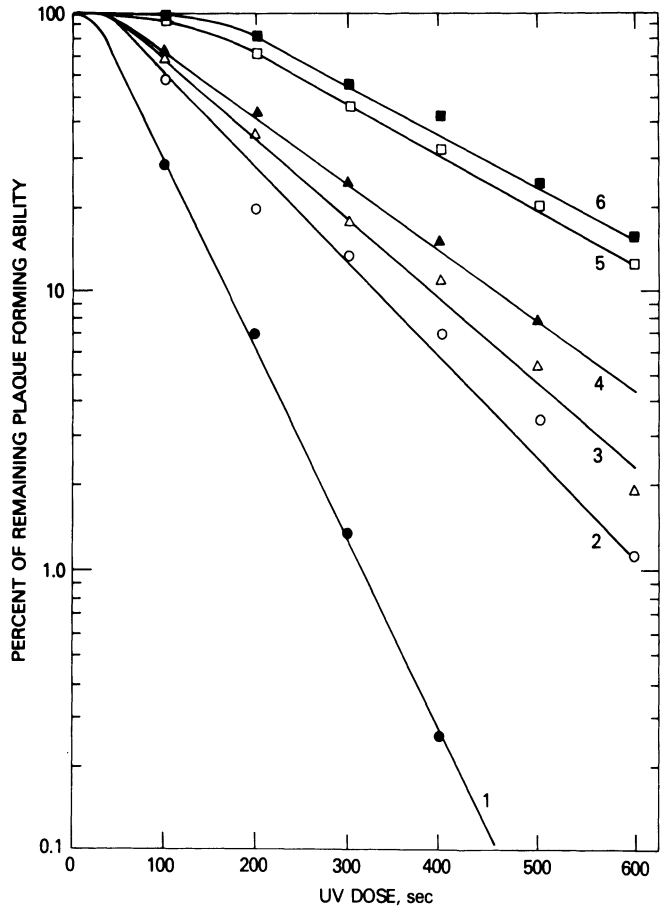


Fig. 7. Survival of λ*gv* as a function of UV dose to the phage prior to adsorption: (1) plated on *E. coli* K12S; (2) plated on UV-irradiated K12S (0.1% survivors), (3) adsorbed on K12(λ), plating infectious centers on K12(λ), (4) adsorbed on K12(λ), plating infectious centers on K12S; (5) adsorbed on UV-irradiated K12(λ), plating infectious centers on K12(λ), and (6) adsorption on UV-irradiated K12(λ), plating infectious centers on K12(λ), and counting only plaques having either *g* or *v* character. (From Jacoband Wollman, 1953, with permission.)

repair-deficient mutants, prophage reactivation was observed in *uvrA*, *uvrB*, *uvrC*, *recB*, *recC*, *exrA*, and *lexA* lysogens, but did not occur in a *recA* lysogen. It also occurred in wild-type *E. coli* in which λ was lysogenized as a plasmid. Using as lysogens five prophages carrying left-arm deletions of various lengths, prophage reactivation of UV-treated λ was found to decrease in a fairly linear way with increasing deletion length. Extrapolation of the line would predict zero prophage reactivation at a deletion length which would leave only the right half of the integrated prophage DNA, suggesting specificity of the recombination event that leads to reactivation. This line of experimentation would

seem to merit further work. Unlike the inducible phenomena (λ induction, WR, and WM), prophage reactivation does not require *lex* (*exr*) function, in agreement with the fact that *lex* is known not to be involved to a great extent in recombination.

Blanco and Devoret (1973) did very similar experiments. These authors also established the dependence of prophage reactivation on *recA* function (and its independence of *lex* function), but found significant prophage reactivation (60% of that occurring in a wild-type lysogen) due to *recA* strains carrying two integrated prophage. Such reactivation was abolished (reported to be ≤ 0) if the infecting irradiated λ were *int*$^-$ *red*$^-$ (a *red*$^-$ alone was not studied), showing that prophage reactivation can also be accomplished by phage genes known to promote recombination. (WM was not observed among the phages reactivated by prophage reactivation unless, of course, the lysogenic host received UV treatment prior to infection.) Thus prophage reactivation is due to constitutive host or phage recombination functions, not on the recombination-dependent, UV-inducible functions.

2.7. Photoreactivation

Photoreactivation of lethally UV-irradiated phage was first reported by Dulbecco (1949, 1950) for the T series of phages. Weigle (1953) showed that the effect also occurred in the case of λ phage, a 3-hr visible fluorescent light treatment of irradiated λ phage *E. coli* K12S complexes reducing the effective UV dose by about a factor of two. When UV-irradiated λ phage were first adsorbed to and plated with *E. coli* K12S which had been irradiated to produce maximal Weigle reactivation and the complexes were subsequently subjected to the 3-hr photoreactivating treatment, little but some photo-induced reactivation (beyond that obtained by Weigle reactivation alone) was observed. The proportion (2.5%) of clear plaque phage mutants produced by Weigle mutagenesis among the surviving λ phage was reduced by photoreactivation of the infected complexes. When the UV-irradiated *E. coli* K12S were subjected to photoreactivation prior to infection with UV-irradiated λ phage, neither reactivation of the phage nor clear plaque mutant production was observed. Thus photoreactivation of irradiated bacteria prior to infection reversed UV-produced alterations responsible for both Weigle reactivation and Weigle mutagenesis, but photoreactivation after infection of the irradiated bacteria gave rise to greater survival of irradiated λ than could be accounted for by Weigle reactivation alone, suggesting that photoreactivation

reversed some UV damage in the phage that Weigle reactivation did not.

2.8. UV Sensitivity and UV Mutagenesis of Lysogenic *E. coli*

The UV resistance of a lysogenic *E. coli* strain is usually somewhat less than that of the nonlysogenic strain, presumably because the induced lytic phage cycle kills the bacterium (Witkin and Wermundsen, 1977; Eitner, 1977). Certain clear plaque mutations of λ, which are believed to result in lower affinity of the λ repressor for λ DNA, can sensitize the cells to UV killing to a much greater extent (Lieb, 1964). In the case of the *cI* 857 *ind*$^-$ repressor, however, a short thermal induction (8 min, 42°C), resulting in the production of many λ early proteins (including the *red* gene products) and little loss of viability, results in *greater* UV survival of UV-treated wild-type, *recB*, *recB recC*, *recB recC recF*, *recB recC recL*, but not *recA* strains (Braun and Gluck, 1977). The effect does not occur if the prophage are mutant at *red*. The *red* product thus appears to suppress the *recB recC* mutation.

3. UV EFFECTS ON BACTERIOPHAGE T4 (T2 AND T6)

The excellent reviews of Stahl (1959) and Friedberg (1975) are recommended. Although the former is more than 20 years old, it gives one of the clearest, most concise accounts available of the application of target theory to the results of Luria-Latarjet multiplicity reactivation and cross-reactivation experiments.

3.1. T4 as a Phage

The T-even phages are moderately unique in that their DNA contains 5-hydroxymethyl cytosine instead of cytosine (Wyatt and Cohen, 1953). This base is often glucosylated (100% in T4, 77% in T2; Sinsheimer, 1956): The T4 gene 56 product is a dCDP-dCTP-ase which converts these nucleotides to dCMP (Wiberg, 1967; Munro and Wiberg, 1968). The T4 gene 42 product converts dCMP to dHMCMP (Flaks and Cohen, 1959; Wiberg *et al.*, 1962; Wiberg, 1967), which is processed to dHMCDP by the T4 gene 1 product (Duckworth and Bessman, 1967). A host kinase is believed to phosphorylate dHMCDP and the triphosphate is incorporated into DNA. Glucosylation utilizing

uridine diphosphoglucose (UDPG) follows. If *E. coli* defective in both B-specific restriction and UDPG phosphorylase are infected by T4 phage having defective genes 56, *denA*, *denB*, and *alc*, viable T4 containing cytosine in their DNA are produced (Runnels and Snyder, 1978). Study of such phage can be expected to lead to an understanding of the role of glucosylated dHMC residues in the 254-nm photobiology of T4 phage.

A fairly current T4 genetic map containing the positions of the genes discussed here was presented by Wood and Revel (1976) and in Volumes 7 and 13 of *Comprehensive Virology*.

3.2. Origins of the Study of UV Effects on T-Even Phage

3.2.1. Growth Delay and Multiplicity Reactivation

In 1944, Luria described a growth-delaying effect of UV light on phages T1, T2, and T7 (then α, γ, and T7). In the case of T1 and T7 phages, a UV dose giving 1% survival increased the time between infection and progeny phage liberation by twofold. With T2, much more sensitive to inactivation of plaque-forming ability, UV produced little if any delay. Later, Luria (1947), while measuring the relative UV sensitivities of various T phages, found that when the survival of the T-even (but not of T5) phages was decreased 4 or 5 orders of magnitude by UV, the remaining survivors appeared much more resistant to further UV inactivation. Further, when the multiplicity of infection by the UV-damaged phages was increased, the plaque counts increased far more than proportionately (unlike nonirradiated phages), suggesting the occurrence of some mutually beneficial intracellular interaction between otherwise inactive phages. The phenomenon, first called cross-reactivation by Luria, is now called multiplicity reactivation. It was reported to occur within the T-even group of phages, but not outside it. For example, damaged T6 (but not T1 or T7) was able to reactivate UV-damaged T2 phage in hosts able to adsorb both phages (but not if the host was unable to adsorb T6). Multiplicity reactivation was hypothesized by Luria to be due to the interphage reassorting of independently transferable phage subunits (many of them damaged) into a viable genetic entity (composed of undamaged subunits).

3.2.2. UV Effects on Developing Phage (Luria-Latarjet Experiment)

It was reasoned by Luria and Latarjet (1947) that, during the production of over 100 phage particles per infected bacterium by a T2-

infected population of bacteria, the survival curves of the T2-infected bacteria as measured by plaque assay (infectious centers) should appear to become more UV-resistant as a function of time after infection, reflecting the increasing number of intracellular phage particles. The study was possible because of the fact that the host's ability to provide adequate biochemistry for supporting T-even phage growth (capacity) was very resistant to UV-irradiation. If there had been no interaction between developing progeny phage particles within a cell, and if the UV-sensitive target for each developing progeny phage were the same as for free phage, then the shape of the survival curve of surviving infectious centers *vs.* UV dose was expected to be both interpretable using the known sensitivity of free phage, and much more resistant than that for free phage, reflecting the necessity for killing all progeny in an infected bacterium in order to prevent that bacterium from giving rise to a plaque on a lawn of noninfected bacteria. Surviving infectious centers would be obtained even if only one progeny phage particle in an infected bacterium survived. While, in general, the expectation of increased UV resistance during the latent period was realized, the comparison of the curves obtained with calculated interpretive curves showed great variance. Further, survival curves obtained under conditions when nonirradiated cells were multiply infected with UV-irradiated T2 (a condition where the UV-sensitive target was the same as for free phage) were also at variance with expected values. These confusing results caused less adventurous workers to note that experiments with radiation always created more problems than they solved. The more adventurous realized that they had a biological phenomenon to unravel.

3.2.3. UV Mutagenesis of T2 Phage

Latarjet (1949, 1954) found that UV caused mutations in phage (T2). Mutations conferring on T2 the ability to grow in an *E. coli* B mutant B-2 (resistant to infection by wild-type T2) were measured. Only irradiation of infected bacteria was mutagenic to T2. Preinfection irradiation of T2 or of the bacterium produced none.

3.2.4. Photoreactivation

Dulbecco (1949, 1950) showed that postinfection near UV (wavelengths near to the visible wavelengths) irradiation of *E. coli* infected by

germicidally 254-nm UV-irradiated T phage increased phage survival. The mercury lines at 365 and 405 nm were noted as particularly effective. Phage T2 was photoreactivated with single-hit kinetics. With phage T2, 56% of the otherwise lethal 254-nm damage could be erased by such treatment. But with the twofold were UV-resistant phage T4, only 20% could be removed. The difference turned out to be due to a gene product (Endo V) carried by T4, not by T2 phage, that aids in repairing UV-damaged T-even phage, and reverses photoreactivable damage in the absence of illumination.

3.3. T4 Phage Genes Governing UV Sensitivity

3.3.1. The *v* Gene

3.3.1a. Characterization and *v* Gene Mutants

One of the processes most effective in repairing UV-damaged phage λ, host-cell reactivation, was found to have no effect on the survival of UV-treated T4 (Ellison *et al.*, 1960). The reason for this is still not known. Luria (1947) found the UV sensitivity of T2 to be twice that of T4. Later, Luria (1949), in studies on the products of crosses of T2 with T4, first recognized phage UV resistance (vs. sensitivity) to be a heritable character. Streisinger (1956) found the character for UV sensitivity, which he called "u^+", to be closely linked to the r_{17} locus. The classification *u* was used to designated UV-resistant phage. Streisinger saw three possibilities to explain the phenomenon: u^+ phages might have an extra length of DNA and, thereby, be more sensitive to UV inactivation; the u^+ locus could be the same size as the *u* locus, but inherently more UV sensitive; or the observed difference in UV sensitivities might be controlled by the u locus: the u^+ allele could inhibit, or the *u* allele could stimulate, repair.

Harm (1961) performed a series of biological experiments in which it was determined that the product of the *u* allele (but not of the u^+ allele) was responsible for reactivation (Fig. 8). Heavily UV-irradiated T2 phage (reproductively inactive) did not change the shape of a UV survival curve of lightly UV-damaged singly infecting T2 phage (when both populations of phage were used to infect *E. coli* B simultaneously), but heavily irradiated T4 phage could increase the survival of T2 phage to near T4 levels. Heavily irradiated T4 phage had very little effect on the shape of the survival curve of lightly irradiated T4 except for a small effect explainable by the expected extent of multiplicity reactivation.

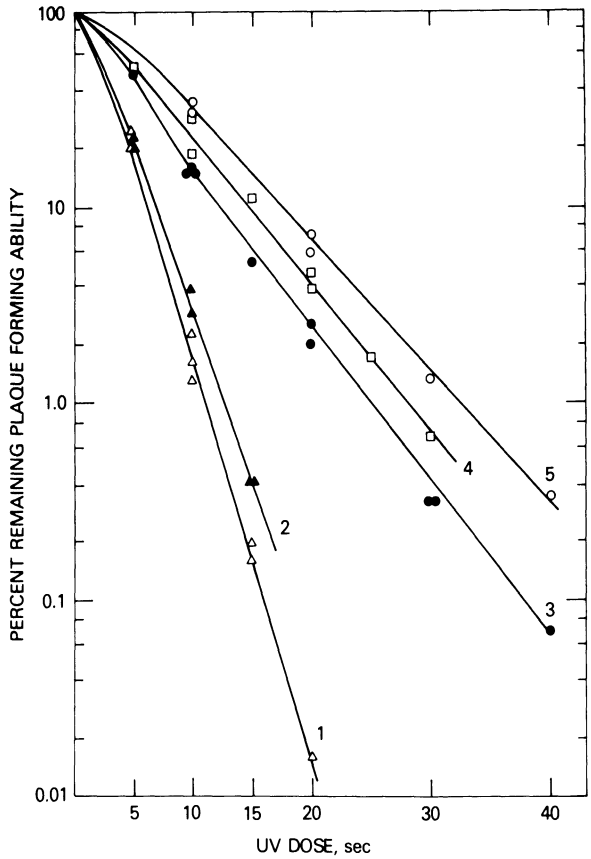


Fig. 8. Survival of T4 (or T2) phage as a function of UV dose. (1) T2 phage infecting *E. coli* B. (2) T2 phage infecting *E. coli* B simultaneously infected with heavily irradiated (5 min) T2, the latter at an m.o.i. of about 3. (3) T2 phage infecting *E. coli* B simultaneously infected with heavily (5 min) irradiated T4, the latter at an m.o.i. of about 3. (4) T4 phage infecting *E. coli* B. (5) T4 phage infecting *E. coli* B simultaneously infected with heavily (5 min) irradiated T4, the latter at an m.o.i. of about 3. Redrawn from Harm (1961) with permission.

Individually picked plaques due to the increased survival of T2 by coinfection with replicatively inactive T4 behaved in their UV sensitivity as T2 phages, showing that such increased survival was not due to donation of an intact *u* gene to the u^+ genome. Harm estimated the size of the *u* gene as 0.9% of the T2 genome by irradiating the already replicatively inactive T4 populations for longer times and measuring their ability to reactivate lightly irradiated T2. The slope of this curve was 0.9% of that of UV inactivation of free T2 phage. The results of Harm's experiment excluded the first two of Streisinger's hypotheses and strongly supported the idea that the function provided by the *u* allele was an enzyme that could repair UV-damaged phage. Soon after,

Harm (1963*b*) reported the isolation of three independent UV-sensitive T4 mutants, T4*v1*, T4*v2*, and T4*x*, from nitrous-acid-treated phage stocks. In this publication, viewing T4 as wild type, he suggested renaming the *u* gene, calling it the *v* gene, designating v^+ as the wild-type state, and using *v* to refer to phage defective in functional reactivation. The UV sensitivities of T4*v1* and T4*v2* were very much like that of T2 and, further, no v^+ recombinants could be obtained in crosses of T2 with T4*v*. T4*x* was somewhat more UV resistant than T4*v* and formed x^+v^+ recombinants with it. Further, the T4*v1x* double mutant was isolated and found to be more UV sensitive than either T4*v1* or T4*x*. When cells infected with UV-irradiated T4, T2,T4*v1*, or T4*v2* were subjected to photoreactivation by visible light, the survival curves became nearly identical, indicating that photoreactivation and the *v* gene product act on similar damage. However, the relatively low survival of maximally photoreactivated T4*x* or T4*v1x* indicated that photoreactivation of these mutants was inefficient, indicating that the *x* and *v* products act to repair different kinds of damage. Since that time, both amber and opal mutants (Van Minderhout *et al.*, 1975) and temperature-sensitive mutants (Sato and Sekiguchi, 1976) and the *v* gene have been characterized.

3.3.1b. Dimer Excision and the Question of *v* Gene Product Packaging

Setlow and Carrier (1968) performed experiments indicating that, during infection of *E. coli* B or Bs-1 by ultraviolet-irradiated $T4v^+x^+$ or $T4^{v+}x^-$ (but not $T4v^-x^+$ or $T4v^-x^-$), thymine dimers are selectively removed from the phage DNA. This process was blocked by 0.01 M KCN or 400 ug/ml chloramphenicol (but not by 100 μg/ml), or by starvation in nonnutrient medium. Shames *et al.* (1973), showing that 150 μg/ml of chloramphenicol added immediately prior to infection of *E. coli* B23 with UV-irradiated T4 blocked protein synthesis, but did not block the UV dose-dependent and *v*-gene-specific nicking of T4 DNA, concluded that *v* gene product was injected along with the DNA by the phage. Pawl *et al.* (1976) reported that 150 μg chloramphenicol per ml blocked the postinfection excision of dimers from UV-irradiated T4. A coinfection by wild-type T4 (nonirradiated) and T4*v* (UV irradiated) resulted in nicking of the T4*v* DNA, whereas, without the wild-type T4, there was none. To produce the hypothetical T4*v* DNA in $T4^+$ coats, a mixed progeny lysate from a 1:1 mixed (total m.o.i. = 10) infection by T4 and T4*v1* was prepared. Survival curves of progeny from individual plaques due to the lysate of progeny showed v^+ and *v* phage to

be in equal quantities. Infection at an m.o.i. of 0.2 with the lysate gave rise to nicks in some, but not all, of the DNA. The conclusion was that multiple copies of the *v* gene product had to have been incorporated into each phage head to account for the data. However, work by Chiang and Harm (1974) provided evidence against this idea. Mixed lysates were prepared as by Shames *et al.* (1973). The authors reasoned that UV irradiation of this mixed lysate should either select strongly for T4v^+ survivors if repair were due to *v* gene product made after infection or should not select very much at all for the v^+ genotype if multiple copies of the *v* gene were injected along with the phage DNA. The results strongly favored the first idea, 360 of the survivors of UV being found to be v^+ as opposed to none being v^- (54 of the 360 phage were expected to be v^- on the basis of the second idea). Further evidence against the phenotypic mixing concept was provided by Van Minderhout *et al.* (1974), who isolated amber (and opal) *v* gene mutants. With respect to UV resistance, such mutants are phenotypically v^- or v^+ when infecting su^- or su^+ strains, respectively. T4*amv* propagated in *E. coli* 63su^+ would have been expected (by the phenotypic mixing hypothesis) to have incorporated active *v* gene product into the phage particles. Its UV resistance when infecting *E. coli* B su^- should then be greater than that of the same phage propagated in *E. coli* B su^-. In fact, irrespective of the su^+/su^- condition of the host used to prepare phage for subsequent irradiation, they showed the same UV sensitivities using the su^- strain as host for measurement of survival. Carlson and Kozinski (1974), however, concluded that the few light parental T4*v* particles which were selected from lysates of BUdR-labeled cells coinfected by BUdR-labeled T4v^+ and light T4*v*1 particles had been packaged with v^+ gene product. They found that these light particles had a UV sensitivity as great as that of v^+ phage. Neither the technical procedure nor the assumptions made in this last report are as straightforward as those of Chiang and Harm (1974) nor of Van Minderhout *et al.* (1974). It is likely that the *v* gene product is not packaged as part of the virion. To clarify the situation, the Shames experiment might be done with 400 μg/ml chloramphenicol. Studies with antibodies specific for the *v* gene product would also be useful.

3.3.1c. The *v* Gene Product

An extensive review of the *v* gene product is given by Friedberg (1975). Only a summary is given here. Initial studies on the *v* gene product, now called endonuclease V, were reported by Takagi *et al.*

(1968), Friedberg and King (1969), Sekiguchi *et al.* (1970), and Yasuda and Sekiguchi (1970*a*,*b*). It was shown that extracts of T4- (but not T4v^-) infected *E. coli* contained an activity that acted specifically on UV-irradiated T4 or *E. coli* DNA, as was anticipated from the results of the biological experiments of Harm (1968). Thymine dimers were found to be preferentially released. The activity produced single-strand breaks if the UV-treated DNA were single stranded (Yasuda and Sekiguchi, 1976), but not if the DNA were not irradiated either single or double stranded (Yasuda and Sekiguchi, 1970*b*; Friedberg and King, 1971). Further, it was found to act on the UV-damaged strand of DNA duplexes made from irradiated and nonirradiated single strands of λ DNA (Simon *et al.*, 1975). Studies with 14 mutants carrying temperature-sensitive mutations at the *v* gene showed that the *v* gene is the structural gene for endonuclease V (Sato and Sekiguchi, 1976; Sekiguchi *et al.*, 1974). The enzyme produces strand incisions on the 5′ side of thymine dimers, creating 3′-hydroxyl and 5′-phosphate termini at the nicking site (Minton *et al.*, 1975).

Pawl *et al.* (1976) found that, on infection of *E. coli* dimer, excision from UV-irradiated T4$^+$ occurred biphasically, a rapid initial loss of 50% of the dimers occurring within 5 min after infection, followed by a very much slower release thereafter. Fewer dimers were released if the bacterial strain infected carried either of two *pol*A polymerase mutations. Further, host mutants at *pol*A deficient in the 5′→3′-exonuclease activity were unable to effect the release of dimers from T4 DNA which had presumably been incised by T4 endonuclease V. Endonuclease V replaced the *uvrA*, *uvrB*, and *uvrC* functions of *E. coli* (Taketo *et al.*, 1972; see also Harm, 1968). The enzyme does not attack DNA damaged by 4-nitroquinoline-*N*-oxide, mitomycin C, nitrogen mustard, or psoralen plus near-UV light (Friedberg, 1972; Ito and Sekiguchi, 1976; Makino *et al.*, 1977), but does act on UV-irradiated DNA in which uracil replaces thymine (Makino *et al.*, 1977).

3.3.2. The *x* Gene(s)

The first T4*x* gene mutant was obtained as described above by Harm (1963*b*). This mutant was found to confer somewhat less UV sensitivity than did the *v* gene to the phage carrying it, and was found not to be closely linked with the *v* gene. The T4v_1x double mutant was found to have twofold increased UV sensitivity over that of phage carrying v_1 alone. Thus the *v* and *x* genes controlled independent repair processes.

Mortelmans and Friedberg (1972) found T4*x* to be approximately threefold more sensitive (on the initial part of the survival curve) to killing by methyl methane sulfonate (MMS) and somewhat (less than a factor of 2) more sensitive to killing by X-rays than T4v_1, which showed wild-type T4 X-ray sensitivity. Further, *x* gene function could not substitute for *recA* gene function in UV-irradiated *E. coli*. T4*x* was also somewhat sensitive to killing by ethyl methane sulfonate (Ray *et al.*, 1972). On the basis of results from cross-reactivation experiments. Harm (1964) postulated that the *x* mutation may either be involved in recombination or be a multisite mutation. Actually, both hypotheses were correct. Drake (1973) separated Harm's *x* mutant into two mutations, *px* (pure *x*) and *hm* (hypermutability). The gene *px* was found to confer sensitivity to UV and MMS and was associated with slow growth and low recombination rates, whereas *hm* conferred none of the above but did effect greater spontaneous and induced mutation rates. Later, Hamlett and Berger (1975) separated out (from Harm's T4*x*) a mutation conferring UV sensitivity which they called x_m (*m* for minute plaque). It is not clear whether the *px* site is the same as the x_m site, but the phenotypic differences caused by the two are nearly the same.

A locus *fdsA*, identified by Dewey and Frankel (1975) as the site of three mutations resulting in the suppression of the lethality of either ts or amber gene 49 (involved in DNA processing and in capsid maturation) mutations, was later found to be the locus of the *x* gene (Cunningham and Berger, 1977).

3.3.3. The *y* Gene

The *y* gene was the name given by Boyle and Symonds (1969) to a locus defined by an amber mutant, y_{10}, having the following properties: sensitivity to UV-produced killing twice that of wild type, normal photoreactivability, 30% more sensitivity to gamma ray killing, slightly reduced multiplicity reactivation when UV'd, and about half the normal recombination frequency. In the accompanying paper, Boyle (1969) demonstrated both successful complementation among phage mutant in genes *v*, *x*, and *y*, and the dominance of the wild-type allele in each case. Locus y_{10} was found to map between genes 24 and 25 by Maynard-Smith and Symonds (1973*b*), who separated from y_{10} a conditionally lethal amber mutation, y_{100}, and concluded that the original mutant was a double mutant, one mutation in an essential gene and the other in an unlinked suppressor of the first. Hamlett and Berger (1975), however, provided evidence for their idea that y_{10}, in addition to y_m (*m*

for minute plaque), contains a suppressor that results in increased plaque size. The y_{100} strain was found to contain, in addition to the y_m and suppressor mutations, another that, in combination with y_m, is lethal. T4y_m has both the original amber UV sensitivity and the decreased recombination frequency characteristic of y_{10}.

In addition to the *x* gene, the *y* gene locus was found also to be a site for mutations (*fdsB*) giving rise to the suppression of the lethal effects of gene 49 mutations (Dewey and Frankle, 1975; Cunningham and Berger, 1977).

3.3.4. Mutant 1206: Gene *w* (*dar*)

Van den Ende and Symonds (1972) isolated a T4 mutant (1206) having a four- to sevenfold reduction in recombination frequency. 1206 was selected from one of five plaques (due to a mutagenized lysate of an *rII* diploid phage), each of which produced haploid segregants at a rate fourfold lower than did the original phage. It was characterized as having at least two mutations outside the *rII* region. While 1206 was only 1.2-fold more UV sensitive than wild-type phage in single infections, it gave rise to a very small shoulder in multiplicity reactivation experiments, behaving similarly to T4*x* in this regard (Symonds *et al.*, 1973). 1206 was found to contain a mutation, *m107*, at gene *w* (Cunningham and Berger, 1977), which was defined by two mutations also resulting in decreased recombination (Hamlett and Berger, 1975). Phage carrying *w* mutations give rise to minute plaques, are recombination deficient, are somewhat sensitive to UV inactivation and to MMS inactivation, and often do not form plaques in the presence of hydroxyurea (30 mg HU added to standard top agar). As pointed out by Cunningham and Berger (1977), the *dar* mutant of Wu and Yeh (1975) may define the same gene as defined by the *w* mutations *m22*, *m33*, and *m107*.

3.3.5. Gene 58

Hamlett and Berger (1975), in selecting T4 mutants from a proflavin mutagenized stock of T4D, found that seven of nine UV-sensitive isolates had both increased UV sensitivity (about 1.7-fold) and increased recombination frequency (four- to ninefold). All seven mapped

in gene 58. Gene 58 is one of four genes that, when nonfunctional, gives rise to a delay in the onset of normal rates of DNA synthesis. (The other genes are 39, 52, and 60; Yegian *et al.*, 1971). Parental DNA in a gene 58 infection was largely nicked, and progeny synthesis occurred about threefold more slowly than in a wild-type infection. The degradation (9–10% acid soluble) was most likely due to exonucleolytic attack by the gene 46–47 controlled nuclease. Like Boyles' *y* mutant, amber gene 58 mutants are UV sensitive or UV resistant when assayed on su^- or *suI* strains, respectively. Because the addition of a mutation in gene 58 to phage already carrying the *v* mutation (but not to phage having the *x*, y_m, or *w* mutations) further sensitized the phage to the lethal effects of UV, it was concluded that the gene 58 repair pathway was independent of that represented by the *v* gene and was part of that represented by the *w*, *x*, and *y* mutations. A gene 58^- infection is accompanied by the overproduction of (autoregulated) gene 32 protein (Krisch *et al.*, 1974), a protein identified earlier by Alberts and Frey (1970) as a single-stranded DNA-binding protein, and known to be required for both recombination and DNA synthesis.

3.3.6. Gene 59

Wu *et al.* (1975) found that two gene 59 mutants, *amC5* and *amHL628*, were 1.5–2.0-fold more sensitive than T4D to the killing effect of both UV and MMS. Later, Cunningham and Berger (1977) found the *amC5* (gene 59^-) mutation to be a double mutant, one of the mutations causing the gene 59 mutation to be lethal. On purification, phage mutant at gene 59 plated with only 90% efficiency on su^- host bacteria, but retained the phenotype of arrested DNA synthesis (Wu *et al.*, 1972) and sensitivity to UV. The addition of a gene *w* mutation to a gene 59 mutant resulted in the suppression of the phenotype of arrested DNA synthesis (as does prolonged chloramphenicol treatment, Wu *et al.*, 1972), but only a slight shift of the UV sensitivity toward that of T4D (Cunningham and Berger, 1977). Similar suppression of gene 59 by *dar-1* was also observed (Wu *et al.*, 1975). Wu and Yeh (1978*b*) showed that biologically inactive 63 S DNA was made during a gene 59 infection. In a *dar* suppressed gene 59^- infection, no 63 S DNA appeared, but a 200 S intermediate (seen in T4D infections) did appear. The *dar* mutant maps in the same region as *w*, between genes 24 and 25 (Wu and Yeh, 1975; Maynard-Smith and Symonds, 1973*b*, Cunningham and Berger, 1977).

Wu and Yeh (1978*a*,*b*) reported that, during infection by a *dar* mutant, the synthesis of gene 32 protein does not undergo the normal shutdown. Further, there are both abnormally high amounts of an 800 S double-stranded DNA intermediate and abnormally low amounts of DNA (analyzed as single strands) of length longer than unit phage DNA produced during T4*dar* DNA synthesis. Moreover, packaging of DNA into heads is delayed.

The phenotype of delayed onset of normal DNA synthesis in gene 39, 52, and 60 amber mutants may be due to defective T4 gyrase or to defective T4 adaptation of *E. coli* gyrase for T4 DNA synthesis (McCarthy, 1979). The observed delay in onset of DNA synthesis in gene 58, 59 mutants may, therefore, reflect defective T4 functions normally concerned with DNA synthesis.

3.3.7. Other Phage Genes Involved in Repair of UV Damage

Baldy (1968, 1970) reported a study of the UV sensitivity of conditionally lethal temperature-sensitive phages mutant at various genes. UV survival curves of mutant phage were done at various increasing plating temperatures with the idea that mutants whose temperature-sensitive lethal defect also affected repair might become more sensitive to UV killing as the temperature increased to the nonpermissive temperature. In this way, genes 30 (ligase), 32 (gene 32 protein), 41 (?), 42 (dCMP-HMase), 43 (DNA polymerase), 44 (?), 45 (?), 46 (DNase), and 47 (DNase), but not genes 45 or 56, were implicated in the repair of UV damage. The UV survival of temperature-independent revertants of the mutants so implicated showed less variation in survival with temperature. Some mutants, among them *tsB20*, *tsL67*, and *tsA52* (genes 30, 32, and 47, respectively), showed UV sensitivity at all temperatures above 24°C. Among the mutants, a certain gene 43 DNA polymerase mutant, *tsL91*, showed a 10% increase in UV sensitivity at 41.5°C.

Speyer and Rosenberg (1968), however, using conditions with which plaque size was increased, found no increased UV of the same mutant at 40.8°C. In following up this discrepancy, Schnitzlein *et al.* (1974) devised two methods to increase plaque size and showed that the "missing plaques" could be restored by using new plating techniques and that tsL91 was not UV sensitive at 42.5°C. However, some T4 carrying some gene 43 mutations (including *tsL91*), but not all, show increased sensitivity to killing due to EMS treatment (Ray *et al.*, 1972).

3.4. Host Gene Functions Involved in the Repair of UV-Damaged T4: *polA*

The use of *E. coli* DNA polymerase (*polA*) mutants as hosts to support the growth of UV-treated wild-type T4 (but not T4*v*) was found by Maynard-Smith *et al.* (1970) to give rise to less phage survival than the use of wild-type hosts. The final inactivation slope was about 30% steeper in $polA^-$ than in $polA^+$ hosts. This finding was confirmed by Wallace and Melamede (1972), suggesting that *E. coli* DNA polymerase I acts in the same repair pathway as the *v* gene product, endonuclease V. Further, like wild-type T4, T4*x* also showed less UV survival using $polA^-$ than on $polA^+$ hosts (Wallace and Melamede, 1972), a behavior expected if the *x* gene product and the Kornberg polymerase work in different repair pathways.

3.5. Further Studies on T4 254-nm Photobiology

3.5.1. Multiplicity Reactivation

The original experiments showing multiplicity reactivation outlined earlier were done by irradiating free phage first and then infecting nonirradiated cells. By contrast, a few of the more recent studies (e.g., Symonds *et al.*, 1973) have been done by irradiating hosts multiply infected with UV-damaged phage, a method which combines procedures common to both multiplicity reactivation and Luria-Latarjet experiments. Because UV irradiation does induce repairlike pathways in *E. coli*, and because any effects of such host UV irradiation on the intracellular events that may alter the UV-irradiated T4 genome have not been studied systematically, details pertaining to experimental design of multiplicity reactivation experiments will be included in the discussion.

3.5.1a. Target Theory Applied to MR

To account for multiplicity reactivation theoretically, Luria (1947) (see also Luria and Dulbecco, 1949) tried to make the simplest assumptions: that each phage is made of *n* subunits, all genetically different, but of equal UV sensitivity, and that at least one of each kind of subunit had to be present and functional in order for a multiply-infected

complex to give rise to a plaque. The probability of survival of such a given subunit in one phage (by Poisson statistics) is $e^{-r/n}$, where r is the number of lethal hits introduced per phage particle (proportional to dose) as measured under conditions where only one phage infects any given bacterium. Since the probability of inactivation of this subunit is one minus the probability of its survival $(1 - e^{-r/n})$, the probability that no such given subunits survives in a bacterium infected with k phages is $(1 - e^{-r/n})^k$. Then the probability that at least one such given subunit survives is $[1 - (1 - e^{-r/n})^k]$, so that the probability of plaque formation (i.e., that at least one copy of each of the n phage subunits is functional (surviving) in the complex) is

$$[1 - (1 - e^{-r/n})^k]^n \tag{1}$$

The distribution of the number of phages infecting a cell when the average m.o.i. is x, given by Poisson statistics, is

$$P(k) = \frac{x^k e^{-x}}{k!}$$

For each value of k, $P(k)$ gives the fraction of the bacterial population infected with exactly k phages.

$$\sum_{k=0}^{k=\infty} \frac{x^k e^{-x}}{k!} = 1$$

reflecting the boundary condition that the probability of a bacterium being either infected or noninfected is 1. Then the expression for multiplicity reactivation, summing the contribution of cells infected with one phage, two phage, three phage, etc., to the viable (multiplicity reactivated) population is

$$\sum_{k=1}^{k=\infty} \frac{x^k e^{-x}}{k!} [1 - (1 - e^{-r/n})^k]^n$$

3.5.1b. Experimental Evidence

As applied to the UV survival of wild-type T4 infecting *E. coli* B, the theory did not fit the data well. (However, Luria's theory fits the multiplicity reactivation data of Bernstein (1957) for phage Vi of

Salmonella typhi quite well.) Harm (1956), in ruling out the point brought up by Cairns and Watson (1956) (that the filament formation observed in cultures of *E. coli* B might make untenable the assumption that the m.o.i. is calculable from Poisson statistics, and throw off the calculations of expected T4 phage survival), showed that the theory could be applied to the data by assuming that 60% of the UV damage could always be totally reactivated by multiplicity reactivation irrespective of m.o.i. and that the remaining 40% of the phage was composed of subunits behaving as Luria hypothesized. Stahl (1959, see also Barricelli, 1960) showed that a good theoretical fit to Harm's data was obtained, making Harm's first assumption and assuming that n = 3 in the second.

Womack (1965, see Fig. 9) presented cross-reactivation data (see Section 3.5.3) obtained under conditions where multiplicity reactivation would take place, giving credence to the idea that there are three large areas in the T4 genome particularly sensitive to UV inactivation. Similarly, Rayssiguier and Vigier (1977), studying the relative marker presence in progeny phage due to a cross of a UV-irradiated T4D

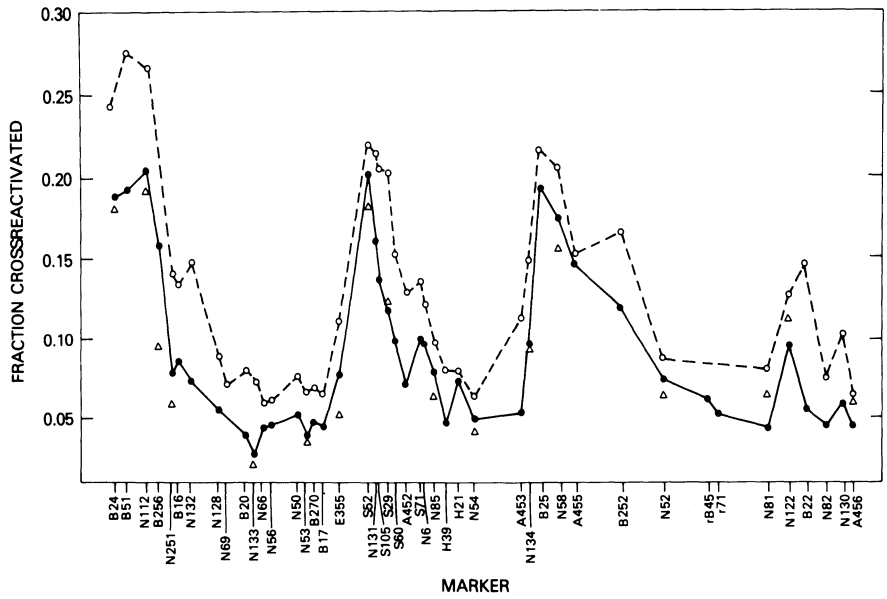


Fig. 9. Cross-reactivation of two rII and 43 amber T4 markers as a function of map distance. The map begins with gene 1 and ends with gene 47. ●, Determined using T4D irradiated with 51 phage lethal hits and using CR-63 bacteria; ○, determined using T4*r71* phage irradiated with 35 phage lethal hits and using CR63 bacteria; Δ, determined using T4D irradiated with 51 phage lethal hits using K112-12 (λh) bacteria. Redrawn from Womack (1965) with permission.

strain, mutant at 18 loci, with a UV-irradiated T4D phage mutant at 20 loci, noted three areas in the genome which were rarely represented in the progeny. Although these authors note a discrepancy between their data and those of Womack at genes 41–46, the total gives some idea that the original ideas of Luria (1947) were not far off target. Barricelli (1960) proposed a partial replica model (several were possible) to account for selective reactivation of certain areas of the genome. A version of this model was favored by Rayssiguier and Vigier (1977) to account for their data. According to their idea, fragments of DNA synthesized from several initiation sites (Huwe *et al.*, 1973; Delius *et al.*, 1971) and terminated (presumably by UV photoproducts) would participate in multiplicity reactivation, thereby increasing the genetic contribution of DNA near these initiation sites. There is evidence that incomplete T4 genomes, having noncircularized DNA, can replicate repetitively (Mosig *et al.*, 1972; Kozinski and Doermann, 1975; see also Kozinski *et al.*, 1976), supporting the proposed mechanism for differential gene representation.

3.5.1c. Phage Mutations Affecting MR

As pointed out by Yarosh (1978), multiplicity reactivation is affected in two ways by bacterial or phage mutations: (1) The final slope of the curve is altered, and (2) the shoulder of the curve is altered. For example, Van den Ende and Symonds (1972) found their mutant 1206 (four- to sevenfold deficient in recombination) to have almost no shoulder in a multiplicity reactivation experiment, although the final inactivation slope was about the same as for wild-type T4. Similar results were obtained in the case of T4*x*, T4*y*, and T4 mutant 1206 (Symonds *et al.*, 1973) or amber mutants having altered gene 32, 44, 46, 47, or 59 (Davis and Symonds, 1974). Curves obtained in similar experiments in which T4*v* (or T4*v* in combination with *x*, *y*, or 1206 mutations) was used to infect wild-type bacteria (Symonds *et al.*, 1973) or in which wild-type T4 was used to infect a *polA* strain (Maynard-Smith *et al.*, 1970) showed markedly increased final inactivation slopes, and either about the same (T4*v*) or increased (*polA*) shoulder extrapolation number. An increased shoulder extrapolation number can be interpreted as an increased number of recombining subunits as follows: taking equation (1) (for the sake of simplicity) and making the approximation $(1 - e^{-r/n})^k \sim 1 - ke^{-r/n}$ at high doses (where the curves become straight lines) and extrapolating that to zero dose we find extrapolation number $= S_{0\ \text{dose}} = k^n$, i.e., the multiplicity of infection to

the power given by the number of recombining subunits. Since the m.o.i. in any one experiment is a constant, the increase in extrapolation number observed by Maynard-Smith *et al.* (1970) would be interpreted as an increase in recombination. Similarly, if the extrapolation number is reduced (it is reduced to zero in the case multiplicity reactivation of gene 59 mutants (Davis and Symonds, 1974)), a decrease in recombination would be indicated by the Luria calculation. Indeed, gene 59 mutants do show a marked recombination deficiency (Cunningham and Berger, 1977). Davis and Symonds (1974) showed that gene 59 mutants (as well as gene 32, 44, 46, and 47 mutants) that are also diploid in the *rII* region segregate phage having haploid *rII* copies at a reduced frequency.

Premier and Chan (1978) have determined that the *recA* product of *E. coli* plays a part in multiplicity reactivation of T4. Using T4D, T4*y*, and T4*x* in a multiplicity reactivation experiment with a *recA* host, very little multiplicity reactivation was noted (compared with the results obtained infecting a rec^+ host), but, similar to the report of Mortelmans and Friedberg (1972), the survival of *singly* infecting UV-irradiated phage was similar to that using T4D to infect AB1157 rec^+ bacteria. Multiplicity reactivation data obtained using irradiated T4D to infect unirradiated AB1157 (Premier and Chan, 1978) were quite similar to those obtained with T4*x*, in contrast to the results of Symonds *et al.* (1973) who used the technique of irradiating phage:host complexes. The difference in methods may therefore be quite important in determining the experimental result.

3.5.2. Luria-Latarjet Curves

The finding by Luria and Latarjet (1947) that UV resistance of phage during development (as measured using the infectious centers assay) increases during phage development led to further studies with other (p8, T7, λ) and related (T2R) phages (Benzer, 1952; Benzer and Jacob, 1953). Ritchie and Symonds (1970) correlated the time at which T4 UV resistance began to develop with the time of onset of T4 DNA synthesis, but noted that, of the two, resistance increased much more rapidly. Simon (1961) showed that chloramphenicol (30 μg/ml) given 4 min after infection slowed the increase in the UV resistance of the phage–host complexes. Further, chloramphenicol added at 9 min after infection led to the production of progeny phage susceptible (without further irradiation) to both photoreactivation and multiplicity reactivation, indicating that the inhibitor blocked complete repair of UV damage

(Tomizawa, 1958). Fisher and Pardee (1968), instead of looking at the production of plaques as an indicator of phage development, used lysozyme synthesis (starting at 10 min after infection) as an endpoint for UV sensitivity. They found a dose of UV, delivered to complexes at times up to 4 min after infection, to be quite effective in reducing the amount of lysozyme produced when the enzyme was measured in samples taken at 11 min of phage development. At 4 min after infection, lysozyme production showed a rapid rise in UV resistance and increasing to a plateau at about 7 min. To account for this phenomenon, which occurred in the absence of detected DNA synthesis, the UV-sensitive target was proposed to be a switch from early to late functions. Other UV-produced phenomena noted to occur in phage-infected complexes include the finding of Krisch and Van Houwe (1976) of greatly overproduced amounts of gene 32 protein (a protein required for T4 DNA synthesis, for recombination (Alberts and Frey, 1970), and for the joining of breaks made in UV'd T4 DNA during repair in experiments done using irradiated complexes (Wu and Yeh, 1973)), and the finding of Prakash *et al.* (1975) of rifampicin-resistant RNA synthesis by UV-irradiated T4 phage–host complexes, but not by complexes made by infecting irradiated host bacteria with nonirradiated phage.

It is probable that the further use of the Luria-Latarjet experimental protocol to study phage:host interactions will lead to a better understanding of the repair processes which affect UV-irradiated T-even phages. It is clear at this point, however, that UV affects the biochemistries of both phage and host, and that a further characterization of the Luria-Latarjet phenomenon, putting well-defined mutants to work, is required. The obvious complexity of the situation should also serve as a warning to those interpreting biochemical data obtained in experiments in which bacteria infected with phage are irradiated during development.

3.5.3. Cross-Reactivation (Marker Rescue) and Survival of Phenotype

"Cross-reactivation" or "marker rescue" is the term now given to a kind of experiment first reported by Doermann *et al.* (1955). The experiment was designed to determine the UV sensitivity of a single given T4 gene function. The experimental design involved infecting the phage host at a multiplicity of 2–3 with a phage mutant at one (or more) genetic site(s). If the cells were simultaneously infected (m.o.i. $<$ 1) with wild-type phage, having the information lacking in the mutant

but lethally damaged by selected (high) doses of UV, the mutant phage may acquire that functional wild-type gene they require if the gene has not been destroyed by the radiation. By single-burst analysis, Doermann *et al.* (1955), using this experimental protocol, determined that the three genes studied each had an inactivation target about 4% the size of that the total phage. They further concluded that inactivation of linked markers occurred more frequently than expected by independent inactivation of each, whereas unlinked markers were inactivated independently.

Krieg (1959*a*) described the results of a study on the *rII* locus in which *E. coli* K12 (λ) was infected at an m.o.i. of 1–5 with an *rII* mutant (nonpermissive infection) and at an m.o.i. of 0.1 of irradiated wild-type T4. The infected complexes were plated on *E. coli* K (λ) to measure rescue of the r^+ gene (cross-reactivation) and on *E. coli* B (5/6) to assay r^+ expression (phenotype) in the mixedly infected K bacteria. The technique eliminated the need for single-burst analysis as Doermann *et al.* (1955) used. Further, it allowed estimation of the survival of phenotype of the wild-type *r* gene (as measured by complementation) whether or not it was rescued by recombination into the mutant phage genome.

Krieg found that two *rIIA* markers were inactivated in the survival of phenotype experiment about twice as fast as were 2 *rIIB* markers, the sensitivity of the *A* and *B* cistrons being 0.1 and 0.05, respectively, of whole phage. The fraction of productive complexes (i.e., in which at least expression of phenotype survived) in which cross-reactivation occurred decreased with UV dose from 1.0 to a constant level between 0.15 and 0.2 so that at the higher doses the inactivation slopes assaying the complexes for cross-reactivation or survival of phenotype were the same.

Doermann (1961) used a slightly different cross-reactivation method, simultaneously infecting *E. coli* B at an m.o.i. of 2–3 with the *rII* mutant and of 0.2–0.6 of the irradiated wild-type phage, finally selecting for r^+ by plating the complexes on *E. coli* K (λ) (see Fig. 10). In this way, UV survival curves of r^+ function with an initial steep portion covering two logs, followed by a linear resistant portion (felt to be due to marker inactivation). This method gave lower estimates for the target size of *rII* markers on the order of 0.006 of the total phage UV target.

Doermann interpreted his extensive data as favoring the idea that two crossovers were required for cross-reactivation. Harm (1961), using a Krieg-type protocol, found that the presence of a *v* mutation in the

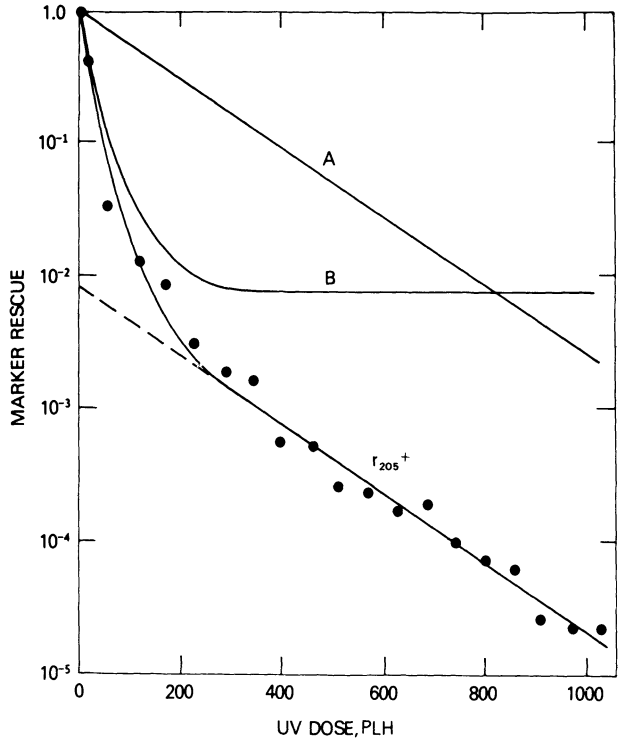


Fig. 10. Marker rescue (cross-reactivation) as a function of UV dose (in phage lethal hits, PLH). Wild-type phage received UV irradiation and were used at an m.o.i. of 0.2–0.6 to infect *E. coli* B simultaneously with T4*r*205 at an m.o.i. of 2–3. After treatment with anti-T4 serum, infected complexes were plated on *E. coli* K12 (λ) prior to lysis. Curve A represents the final slope of the rescue curve. Curve B represents the rescue curve divided by curve A. Redrawn from Doermann (1961) with permission.

phage used to measure the UV sensitivity did not greatly affect the inactivation rate of *rIIB* loci. Further, using a similar design to measure the ability of the T4 *v* gene product to repair UV'd T2 phage, the inactivation slope of *v* gene action was about 1% of that obtained from a survival curve of free phage.

Using the technique of Doermann (1961), Harm (1964) analyzed the effect of including a mutation in the *x* and *v* genes in either the irradiated wild-type phage or in the nonirradiated *rII* mutant or in both. If the nonirradiated *rII* parent were mutant at *x*, the rescue of the wild-type *rII* marker was about twofold more sensitive to UV than if the *rII* parent were x^+. Whether the irradiated wild type bore a wild-type *x* gene (even when the *rII* parent did not) made no difference, presumably because the UV doses required to inactivate the wild *rII* allele

were so great that it was improbable that any phage having a rescuable wild-type *rII* allele also had a functional *x* gene. The slopes of both the initial steep portion of the inactivation and the linear-resistant portion were observed to be greater (indicating more UV sensitivity) in the absence of functional *x* gene product. A similar set of experiments using phage mutant or not in the *v* gene showed that the lack of functional *v* gene led to an increase in slope of the intial steep portion, but not of the linear-resistant portion of the curve.

Harm further found that T4*xrII* mutants had a threefold decrease in recombination frequency when compared to the same *rII* crosses in T4x^+ phage. Thus it would seem reasonable to interpret the slope of the linear-resistant portion of the curve as reflective of the probability of recombination rescuing the marker, given that there are surviving "recombinable markers." The slope of the intial steep portion would reflect the probability that recombinable markers would survive, and therefore the probability that successful recombination would occur at all. (UV delivered to the phage presumably decreases number of progeny DNA units synthesized and, in doing so, also decreases the number of phage recombination events per cell.)

Again using Doermann's (1961) technique, Winkler *et al.* (1962) found the slope of the linear-resistant portion of cross-reactivation curve to be a function of the wavelength of irradiation. Of the eight wavelengths between 225 nm and 295 nm studied, the shallowest (reflecting most resistance) slope was obtained with 245 and 255 nm. The results for each wavelength were plotted as cross-reactivation vs. phage lethal hits due to that wavelength. If the photoproduct(s) responsible for lethality had been the same as that responsible for cross-reactivation, the curves would have been the same. This was not the case. Some photoproduct(s) produced maximally at 245–255 nm were relatively more effective than the photoproducts produced at other wavelengths in producing cross-reactivation (or less effective in inhibiting cross-reactivation) than in killing the phage.

Ebisuzaki (1966) greatly expanded on earlier UV work with T2 phage (Delihas, 1961; Dirksen *et al.*, 1960). He measured *v* gene effects on survival of phenotype by infecting *E. coli* at an m.o.i. of 0.3 or less with previously irradiated phage, and assayed enzymatic activities directly after having allowed sufficient time for their expression. As has been noted previously, T4v^+ phage require twice the UV dose to be inactivated to a given survival as do T4*v* phage. Thus it was somewhat surprising that Ebisuzaki found that the UV sensitivities of the early functions (i.e., gene 1, 42, 56, and 32 products—this last done by a

Krieg-type experiment) were not changed on substitution of T4*v* for T4v^+ in the experimentation. However, the UV depression in synthesis of phage DNA, late proteins, lysozyme, and thymidylate synthetase (all late functions) were *v* gene dependent, as expected. Later, Ebisuzaki (1969) found the intracellular production of thymidylate synthetase after infection by a UV'd T4 gene 43 mutant to be as depressed by UV as if a T4*v* were used; a UV'd gene 30 mutant, however, gave the higher wild-type levels of the enzyme. To account for the data, the gene 43 product (but not the gene 30 product) was postulated to be required for successful repair.

However, in similar experiments that were designed following the procedure of Krieg (1959*a*), Maynard-Smith and Symonds (1973*a*) showed that the UV-depressed expression of genes 32, 41, 43, and 44 (but not that of genes 1, 30, 42, 45, and 56) was as dependent on *v* gene function as was phage survival. The damage resulting in loss of gene 42 function, although not repaired by *v* gene action, was partially restorable by photoreactivation, indicating both that some other non-*v* repair of the phage did not unexpectedly occur and that the *v* gene (or *v* gene product) is nonfunctional in the absence of gene 42 product. Thus Maynard-Smith and Symonds interpreted their results to mean that gene 1, 30, 42, 45, and 56 products were all required for excision repair, but that gene 32, 41, 43, and 44 products were not. Although this interpretation may overreach the data, the expression of the gene 1, 30, 42, 45, and 56 products would certainly seem to be necessary for *v* gene action to lead to successful repair.

There are obvious conflicts in the conclusions of Ebisuzaki (1966, 1969) and of Maynard-Smith and Symonds (1973*a*) regarding the roles of the products of genes 30, 32, and 43 in repair. The conflicts regarding genes 30 and 32 are difficult to resolve without further work. The fact that Maynard-Smith and Symonds' work was done with cells infected at high m.o.i. with mutant nonirradiated phage (in addition to the irradiated phage, which were the only phage Ebisuzaki used) may have given rise to the problem. T4 gene 43 mutants have wild-type UV sensitivity (when assayed for plaque forming ability at semi-permissive temperatures), showing that little if any T4 polymerase is required for repair (Schnitzlein *et al.*, 1974).

Excitement in the area of cross-reactivation has dropped considerably, presumably giving way to the onrush of experimentation in the areas of recombination and mutagenesis. However, the phenomenon has proved a precision tool and would certainly be useful for the further analysis of the role of recombination in DNA repair.

3.5.4. UV Mutagenesis

After the finding of Latarjet (1949, 1954) that UV delivered to infected complexes (but not free phage) produced mutations among the progeny phage, Krieg (1959*b*, high m.o.i. one cycle of growth before selection) and Folsome and Levin (1961, low m.o.i. direct selection) produced *rII* reversions by irradiating T4 phage only.

3.5.4a. Base-Pair Specificity of UV-Mutagenesis of T4

Setlow (1962) found that the action spectrum for T4 inactivation, obtained by irradiation of complexes at 7 min of development, had the appearance of a single-stranded DNA absorption spectrum (a minimum at 240 nm, not 235 nm). She studied the effect of UV of various wavelengths in reverting two mutants studied by Freese *et al.* (1961), and concluded they were due to an A-T mutant base pair. She found the action spectrum for reversion of both mutants to be quite similar to the absorption spectrum of thymidine, thus implicating thymidine (whose absorption in single-stranded DNA would presumably be similar to that of free thymidine) as a target in UV-produced reversion. Unfortunately, no mutant suspected of having a G-HMC mutant base pair was available as a control.

Drake (1963) used the principles of identification of base changes in T4 as worked out by Freese (1959*a*,*b*) and Freese *et al.* (1961) (which Champe and Benzer (1962) had applied to T4 mutagenesis by 5-fluorouracil) to analyze the mutagenic effects of UV (delivered at 8 min of phage development) in the *rII* region. These principles are, briefly, as follows: Transition mutations (arising from a Pu:Py → Pu:Py base pair change) are usually revertible by either BU or 2AP. Hydroxylamine (reacting with HMC) also reverts transition mutants (reverting BU responders more strongly than mutants responding to 2AP), so that if a mutant responded to both BU and HA, the base pair at the mutant site is likely G-HMC. If the mutant does not respond to HA treatment, an A-T mutant site is indicated. Mutants not classified as transition mutants, which revert on treatment with proflavin, are classed as possible missing or extra base mutants (Crick *et al.*, 1961; Orgel and Brenner, 1961). Drake used mutants mapped by Benzer: 302 UV-produced *rII* mutants (belonging to both cistrons) fell into 99 independent sites. Of these, 168 fell into the B cistron (32 and 19 were at two sites at which many spontaneous mutants were also mapped, and 49 were at another site where spontaneous mutants rarely mapped). Mutants in

the *A* cistron did not cluster as greatly. Drake tested UV-induced mutants representative of all 99 sites for reversion, finding 47 to have an A-T mutant base pair, six to have a G-HMC mutant base pair, and 46 to be nontransition sites. Drake also found a large variation in UV-produced reversion among 14 mutants having an A-T mutant base pair.

Drake (1966*a*), studying mutations produced by extracellular irradiation of T4, found multiplicity reactivation not to be required for the UV production of *r* mutants, and that 2×10^{-4} *r* mutants were produced per lethal hit, independent of the presence of the *v* gene. Of about half of the mutants mapped in the "hot spots" alluded to previously, about 60% were revertible by base analogues. Such mutants appeared to be generally similar to those produced by irradiation of developing phage. Photoreactivation (Drake, 1966*b*) of irradiated T4*v* phage–host complexes (blocked in development by KCN) reversed about half the mutants produced, an effect very much like that produced by *v* gene action. (The results of Azizbekyan and Pogosov, 1971, would appear to be in direct conflict with Drake's and are referred to here without further comment.)

Meistrich and Drake (1972), taking advantage of the fact that acetophenone D plus 313-nm light was found to produce almost exclusively thymine dimers in T4 phage DNA (Meistrich and Lamola, 1972), studied the contribution of such photoproducts to T4 mutagenesis. Similar to the case with UV alone, 2.4×10^{-4} *r* mutants were produced per lethal hit by such treatment; nearly all were photoreactivable, as expected if thymine dimers were causing them. However, two-thirds of the mutants were nonrevertible by base analogues, 16% were GC → AT transitions, and only 10% were AT → GC transitions. The conclusion was made that thymine dimers do not act in mutagenesis by direct miscoding during DNA replication, but rather as a premutational lesion. These data would appear to contradict Setlow's (1962) hypothesis that UV-produced reversion is caused by direct action of UV on thymine in T4 DNA. (Certainly reversion could have been produced indirectly in Setlow's experiments by biological reactions following an initial change caused by absorption of UV photon by thymine.)

3.5.4b. Genetic Determinants of T4 Mutability

Drake (1973), while studying phage genes influencing reversion rates, was able to identify the *x* mutant of Harm as a complex composed of *px* (pure *x*, conferring sensitivity to UV and MMS, and reduced recombination rates) and *hm* (hypermutable by UV, having

wild-type UV and MMS sensitivity). He determined that, in contrast to wild-type T4 which in these experiments gave 6×10^{-4} *r* mutants per phage lethal hit, T4*px* gave 0.9×10^{-4} and T4*hm* gave 19×10^{-4}. Green and Drake (1974) determined that the rates of production of *r* mutants in T4 on UV, MMS or psoralen plus white light treatment were not only abnormally low in T4*px* (four- to sevenfold lower) but also in T4*y* (five- to thirtyfold) and in 1206 (25- to 100-fold lower). Mutation rates were 1.7- to 2.2-fold greater in T4*hm* than in wild-type phage. Thus it was proposed that the mutations *px*, *y*, and 1206 defined genes involved in an error-prone recombination-repair system much like the *recA*-dependent system of *E. coli* (Witkin, 1969*a*; Radman, 1974; Witkin and George, 1973). Yarosh (1978) studied the effects of a phage gene 30 (*lig*) mutation on UV-induced reversion of two T4 amber late mutants. By plating the UV'd phage directly after UV on the restrictive host and incubating at 25 or 31°C, a dependence of reversion on gene 30 was demonstrated. The UV-produced revertants obtained at 31°C were not observed when the amber phage was also mutant at gene 30, but, at 25°C, reversion of the phage occurred. Thus functional ligase was implicated in the UV production of revertants. It was demonstrated that the reversion frequency was greatly increased when the irradiated phage was passaged through a permissive host before selection of revertants, indicating that some metabolism requiring late functions was presumably required for the "fixation" of mutations. (It would have been better to use otherwise isogenic restrictive and permissive strains for this comparison, however.) Nevertheless, when Yarosh compared the UV-produced reversion of the mutants obtained under conditions of multiplicity reactivation with that obtained by single infection (passaged through the permissive host in both cases), a far lower reversion frequency was obtained at a given UV dose using the former condition than using the latter. To correct for differences in survival obtained under the two conditions, the data were plotted as reversion frequency vs. surviving fraction, but, even plotting in this fashion, the reversion frequency was still less under conditions of multiplicity reactivation. Yarosh concluded that multiplicity reactivation is an error-free repair pathway and that such reactivation may selectively eliminate mutagenic UV lesions in preference to lethal ones.

3.5.5. UV-Enhanced Recombination

Enhanced recombination among UV-irradiated T4 phage was first noted in a study of four marker pairs by Epstein (1958). Generally, the

dose-dependent increase was more rapid the greater the map distance between the markers. Chan (1975) noted that such stimulation was greater at a given UV dose if both *rII* mutants being crossed also contained a defective *v* gene. Later, Premier and Chan (1978), while confirming this, showed that if both mutants contained Harm's original defective *x* gene, the UV enhancement was less than if the *rII* mutants had wild-type repair.

3.5.6. The Lethal UV Photoproduct(s) in T4 Phage

Pyrimidine dimers are produced in relatively high yields by 254-nm irradiation of T4 DNA. These are subject to direct enzymatic reversal in the presence of light by photoreactivation or to *v* gene-mediated excision, as noted previously. There are several reports indicating that photoproducts not susceptible to *v* gene-mediated repair or to photoreactivation are produced in good yields by 254-nm irradiation of T4 phage. Cavilla and Johns (1964) showed that the ratio of the inactivation slopes of T2 (lacking the *v* gene) and T4 at 254 nm to be 2.4, while at 302 nm the ratio was 4.0. This result would not be expected if there were one photoproduct produced by both wavelengths. Meistrich (1972), using acetophenone D plus 313-nm irradiation to produce mainly thymine dimers in T4 DNA, found that the ratio of the inactivation slope of T4*v* to that of T4 was 4.4, in contrast to 2.2 obtained if the phage had received 254-nm irradiation. The number of dimers produced per lethal hit in the case of $T4^+$ was 10.2 with 254-nm light and 26 with acetophenone D plus 313-nm light. For T4*v*, the numbers were 4.7 for 254 nm and 6.0 acetophenone D plus 313 nm, and for T4*vx* 2.5 and 3.3, respectively. Meistrich concluded that 254-nm irradiation must produce lesions other than thymine dimers, and that both photoreactivation and *v* gene-mediated repair act preferentially on thymine dimers.

4. UV Effects on ϕX174

ϕX174 was identified by Sertic and Boulgakov (1935). The virion was found to contain a single molecule of single-stranded DNA, 1.7×10^6 in molecular weight (Sinsheimer, 1959). Viral replication is accompanied by a chloramphenicol-resistant conversion of the single-stranded viral DNA molecule to a double-stranded structure (replicative form or RF I), which undergoes many rounds of chloramphenicol-resistant duplication (Sinsheimer *et al.*, 1962). Progeny single-stranded DNA is

generated at later times of infection by a chloramphenicol-sensitive process.

4.1. UV Sensitivity of Single-Stranded and RF DNAs

Using the finding that RF molecules as well as single-stranded molecules are infectious in spheroplast assays (Sinsheimer *et al.*, 1962), the single-stranded DNA was found to be about tenfold more sensitive to inactivation by UV than the RF (Sinsheimer *et al.*, 1962). Similar data were obtained by Tessman (1966) for the related phage S13.

It was shown (Jansz *et al.*, 1963; Yarus and Sinsheimer, 1964; Sauerbier, 1964) that the UV sensitivity of the double-stranded form of the DNA closely approached that of the single-stranded DNA if its biological activity were assayed using a strain incapable of excising pyrimidine dimers in place of a host capable of excision repair. The level of reactivation of UV-damaged RF is reduced somewhat in the absence of functional host polymerase I (Klein and Niebch, 1971). After the finding of excision of thymine dimers from UV-damaged DNA (Setlow and Carrier, 1964; Boyce and Howard-Flanders, 1964) and the accompanying hypothesis that successful completion of excision repair required DNA synthesis using the nonexcised strand as a template (supported by the results of Pettijohn and Hanawalt, 1964) it was believed that the reason for the observed high sensitivity of UV-irradiated ϕX174 single-stranded DNA was that it was not subject to successful excision repair because it lacked the repair template. If this were true, then one photoproduct might be expected to be lethal, since attempted excision repair would sever a single-stranded molecule. Indeed, David (1964) measured 0.4 thymine dimers per viral genome at a 260-nm UV dose that introduced on the average one lethal hit per infectious virion. The fact that the action spectrum for viral inactivation more closely paralleled the absorption spectrum of the virus than of its DNA (Setlow and Boyce, 1960; Yarus and Sinsheimer, 1967) suggested that the viral protein (as well as the DNA) might be implicated in UV inactivation of the virion. Evidence implicating DNA-protein cross-links as about 5% of the lethal UV effect has been presented (Francke and Ray, 1972).

4.2. Effects of UV-Irradiating Host Cells

Large UV doses delivered to the host cells before ϕX174 infection blocked DNA synthesis at the chloramphenicol-sensitive step of conver-

sion of RF to progeny single strands (Matsubara *et al.*, 1967). This effect was possible due to the preferentially decreased levels of cistron V product (a protein required for single-stranded DNA synthesis) noted in UV-irradiated cells by Godson (1971). Such blocked conversion also occurred when UV-damaged phage DNA was treated *in vitro* (Masamune, 1976). On the other hand, UV-irradiated phage infecting nonirradiated bacteria are blocked in DNA synthesis at the step of conversion of viral DNA to RF (Poddar and Sinsheimer, 1971) at the site of UV lesions (Francke and Ray, 1971; Benbow *et al.*, 1974; Caillet-Fauquet *et al.*, 1977). The UV lesions responsible for such blockage of chain elongation *in vitro* were removed by light in the presence of yeast photoreactivating enzyme, indicating that these lesions are likely to be pyrimidine dimers (Poddar and Sinsheimer, 1971).

Irradiation of the host cell gave increased survival of UV-irradiated phage (Bleichrodt and Verheij, 1974; DasGupta and Poddar, 1975) as reported previously for the single-stranded DNA phages S13 (Tessman and Ozaki, 1960) and ϕR (Ono and Shimazu, 1966). The reactivation of ϕX174 did not occur in cells deficient in *recA* function, and occurred maximally in *uvrA* hosts at doses about tenfold lower that used for maximal reactivation in the case of wild-type bacteria, paralleling the same phenomenon observed using phage λ. Also paralleling the λ results was the fact that UV reactivation of UV-irradiated ϕX174 and S13 phages was accompanied by a large increase in the mutation frequency (Bleichrodt and Verheij, 1974; Tessman and Ozaki, 1960). UV reactivation of UV-irradiated ϕX174 was also observed in host cells undergoing thymineless death (Thakur and Poddar, 1977). That UV reactivation is successful in the case of single-stranded phages shows that, unlike excision repair, an undamaged repair template is not required for its occurrence. ϕX174 DNA synthesis might be blocked by UV lesions in UV-irradiated cells (as it is in nonirradiated cells, as noted previously) and the observed mutagenesis would be due to miscoding by DNA lesions during replication on a damaged template.

Testing this idea, Caillet-Fauquet *et al.* (1977) compared the ability of UV-irradiated or nonirradiated *E. coli* to support DNA synthesis due to infecting UV-irradiated or control ϕX174. To account for the data obtained, it was proposed that (1) a UV lesion in the single-stranded infecting ϕX174 DNA blocked DNA replication at the site of the lesion if the host *E. coli* had received no UV prior to infection (in accord with the findings of Francke and Ray, 1971, and Benbow *et al.*,

1974), and (2) preinfection irradiation of the host cells permitted a chloramphenicol-sensitive (inducible) conversion of some of the irradiated ϕX174 single strands to RF. Postinfection thymineless starvation also results in conversion of irradiated ϕX174 viral single strands to RF (Benbow *et al.*, 1974).

4.3. Other Studies

UV delivered to ϕX174 prior to infection stimulates phage recombination (Benbow *et al.*, 1974). The effect was abolished if the host cells carried a *recA* mutation, but was also observed when host cells were starved for required thymine after infection by nonirradiated phage.

Luria-Latarjet experiments, in which bacteria where irradiated after various periods of ϕX174 development and then were analyzed as infectious centers, showed curves approximating the expected result (Sections 3.2.2 and 3.5.2; Denhardt and Sinsheimer, 1965), in contrast to T2 phage (Luria and Latarjet, 1947).

5. CONCLUSION

Many of the data pertaining to the effects of UV on phages λ, T4, and ϕX174 have been presented. The effects of the irradiation on the replication of the infecting phage have been analyzed both in terms of the damaging events occurring to phage DNA prior to infection and in terms of the response of repair/recombination mechanisms (host or virion coded) to them. Particular attention has been focused on the biological events (induction of λ, enhanced recombination, multiplicity reactivation, host-cell reactivation, Weigle reactivation, and mutagenesis) that often occur as a result of host metabolism (induced by the UV or not) interacting with that of the phage.

NOTE ADDED IN PROOF. The T4 *v* gene activity that shows specificity in producing breaks near dimers has recently been found to consist of two activities: (1) a glycosylase that separates one pyrimidine of a dimer from its sugar, and (2) an apurinic/apyrimidinic (AP) endonuclease that cleaves on the 3′ side of the AP site (Haseltine *et al.*, 1980; Radany and Friedberg, 1980; Demple and Linn, 1980).

6. REFERENCES

Alberts, B. M., and Frey, L., 1970, T4 bacteriophage gene 32: A structural protein in the replication and recombination of DNA, *Nature (London)* **227**:1313.

Atwood, K. C., and Norman, A., 1949, On the interpretation of multi-hit survival curves, *Proc. Natl. Acad. Sci. USA* **35**:696.

Azizbekyan, R. R., and Pogosov, V. Z., 1971, Repair of UV-induced mutations in phage T4, *Biochem. Biophys. Res. Commun.* **43**:1402.

Bachmann, B. J., Low, K. B., and Taylor, A. L., 1976, Recalibrated linkage map of *Escherichia coli* K-12, *Bacteriol. Rev.* **40**:116.

Baker, R. M., and Haynes, R. H., 1967, UV-induced enhancement of recombination among lambda bacteriophages in UV-sensitive host bacteria, *Mol. Gen. Genet.* **100**:166.

Baker, R. M., and Haynes, R. H., 1972, UV-induced recombination and repair of parental lambda bacteriophage labeled by means of host controlled modification, *Virology* **50**:11.

Baldy, M. W., 1968, Repair and recombination in phage T4. II. Genes affecting UV sensitivity, *Cold Spring Harbor Symp. Quant. Biol.* **33**:333.

Baldy, M. W., 1970, The UV sensitivity of some early-function temperature-sensitive mutants of phage T4, *Virology* **40**:272.

Barbour, S. D., and Clark, A. J., 1970, Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. I. Enzymatic activity associated with $recB^+$ and $recC^+$ genes, *Proc. Natl. Acad. Sci. USA* **65**:955.

Barbour, S. D., Nagishi, H., Templin, A., and Clark, A. J., 1970, Biochemical and genetic studies of recombination proficiency in *Escherichia coli*. II. Rec^+ revertants caused by indirect suppression of rec^- mutations, *Proc. Natl. Acad. Sci. USA* **67**:128.

Barricelli, N. A., 1960, An analytical approach to the problems of phage recombination and reproduction. I. Multiplicity reactivation and the nature of radiation damages, *Virology* **11**:99.

Benbow, R. M., Devoret, R., and Howard-Flanders, P., 1973, Indirect ultraviolet induction and curing in *E. coli* cells lysogenic for bacteriophage λ, *Mol. Gen. Genet.* **120**:355.

Benbow, R. M., Zuccarelli, A. J., and Sinsheimer, R. L., 1974, A role for single-strand breaks in bacteriophage ϕX174 genetic recombination, *J. Mol. Biol.* **88**:629.

Benzer, S., 1952, Resistance to ultraviolet light as an index to the reproduction of bacteriophage, *J. Bacteriol.* **63**:59.

Benzer, S., and Jacob, F., 1953, Etude du developpment du bacteriophage au moyen d'irradiations par la lumiere ultraviolette, *Ann. Inst. Pasteur* **84**:186.

Berstein, A., 1957, Multiplicity reactivation of ultraviolet-irradiated Vi-phage II of *Salmonella typhi*, *Virology* **3**:286.

Bertani, G., and Weigle, J. J., 1953, Host controlled variation in bacterial viruses, *J. Bacteriol.* **65**:113.

Beukers, R., and Berends, W., 1960, Identification of the irradiation product of thymine, *Biochim. Biophys. Acta* **41**:550.

Blanco, M., and Armengod, M. E., 1976, Role of the bacterial and phage recombination systems and of DNA replication in genetic recombination of UV-irradiated phage lambda, *Mol. Gen. Genet.* **146**:51.

Blanco, M., and Devoret, R., 1973, Repair mechanisms involved in prophage reactivation and UV reactivation of UV-irradiated phage λ, *Mutat. Res.* **17**:293.

Bleichrodt, J. F., and Verheij, W. S. D., 1974, Mutagenesis by ultraviolet radiation in bacteriophage ϕX174: On the mutation stimulating processes induced by ultraviolet radiation in the host bacterium, *Mol. Gen. Genet.* **135**:19.

Bonhoeffer, F., 1966, DNA transfer and DNA synthesis during bacterial conjugation, *Z. Vererbungsl.* **98**:141.

Borek, E., and Ryan, A., 1958, The transfer of irradiation-elicited induction in a lysogenic organism, *Proc. Natl. Acad. Sci. USA* **44**:374.

Borek, E., and Ryan, A., 1960, The transfer of a biologically active irradiation product from cell to cell, *Biochim. Biophys. Acta* **41**:67.

Borek, E., and Ryan, A., 1973, Lysogenic Induction, in: *Progress in Nucleic Acids Research and Molecular Biology*, Vol. 13 (J. N. Davidson and W. E. Cohn, eds.), pp. 249–300, Academic Press, New York.

Boyce, R. P., and Howard-Flanders, P., 1964, Release of ultraviolet light-induced thymine dimers from DNA in *E. coli* K-12, *Proc. Natl. Acad. Sci. USA* **51**:293.

Boyle, J. M., 1969, Radiation-sensitive mutants of T4D. II. T4y: Genetic characterization, *Mutat. Res.* **8**:441.

Boyle, J. M., and Setlow, R. B., 1970, Correlations between host-cell reactivation, ultraviolet reactivation and pyrimidine dimer excision in the DNA of bacteriophage λ, *J. Mol. Biol.* **51**:131.

Boyle, J. M., and Symonds, N., 1969, Radiation sensitive mutants of T4D. I. T4y: A new radiation-sensitive mutant; effect of the mutation on radiation survival, growth and recombination, *Mutat. Res.* **8**:431.

Braun, A. G., 1976, Host DNA replication or excision repair requirement for ultraviolet induction of bacteriophage λ lysogens, *Nature* (*London*) **261**:164.

Braun, A., and Gluck, D., 1977, Effect of transient lambda prophage induction in ultraviolet light resistance and recombination in *Escherichia coli*, *J. Bacteriol.* **131**:208.

Brenner, D. J., and Groman, N. B., 1966, Inhibition of coliphage reproduction after superinfection of induced lysogens, *J. Bacteriol.* **92**:1727.

Breseler, S. E., Kalinin, V. L., and Shelegedin, V. N., 1974, Inactivation of transfecting DNA by physical and chemical agents—Influence of genotypes of phage lambda and host cells, *Mutat. Res.* **25**:235.

Breseler, S. E., Kalinin, V. L., and Shelegedin, V. N., 1978, W-reactivation and W-mutagenesis of gamma irradiated phage lambda, *Mutat. Res.* **49**:341.

Bridges, B. A., 1975, An ultraviolet-resistant tsDNA mutant of *Escherichia coli* deficient in host cell reactivation ability for bacteriophage λ and showing hypersensitivity towards induction of Weigle reactivation, *Mutat. Res.* **29**:489.

Brooks, K., and Clark, A. J., 1967, Behavior of λ bacteriophage in a recombination deficient strain of *Escherichia coli*, *J. Virol.* **1**:283.

Buttin, G., and Wright, M., 1968, Enzymatic DNA degradation in *E. coli:* Its relationship to synthetic processes at the chromosome level, *Cold Spring Harbor Symp. Quant. Biol.* **33**:259.

Caillet-Fauquet, P., and Defais, M., 1972, UV reactivation of phage λ in a polA mutant of *E. coli*, *Mutat. Res.* **15**:353.

Caillet-Fauquet, P., and Defais, M., 1977, Kinetics of induction of error-prone repair of bacteriphage λ by temperature shift in an *Escherichia coli* dnaB mutant, *Mol. Gen. Genet.* **155**:231.

Caillet-Fauquet, P., Defais, M., and Radman, M., 1977, Molecular mechanisms of induced mutagenesis. Replication *in vivo* of bacteriophage ϕX174 single-stranded, ultraviolet-irradiated DNA in intact and irradiated host cells, *J. Mol. Biol.* **117**:95.

Cairns, H. J. F., and Watson, G. S., 1956, Multiplicity reactivation of bacteriophage, *Nature* (*London*) **177**:131.

Calvert, J. G., and Pitts, J. N., 1966, *Photochemistry*, Wiley, New York.

Carlson, K., and Kozinski, A. W., 1974, Nonreplicated DNA and DNA fragments in T4r$^-$ bacteriophage particles: Phenotypic mixing of a phage protein, *J. Virol.* **13**:1274.

Castellazzi, M., George, J., and Buttin, G., 1972, Prophage induction and cell division in *E. coli.* I. Further characterization of the thermosensitive mutation tif-1 whose expression mimics the effect of UV irradiation, *Mol. Gen. Genet.* **119**:139.

Cavilla, C. A., and Johns, H. E., 1964, Inactivation and photoreactivation of the T-even phages as a function of the inactivating ultraviolet wavelength, *Virology* **24**:349.

Champe, S. P., and Benzer, S., 1963, Reversal of mutant phenotypes by 5-fluorouracil: An approach to nucleotide sequences in messenger-RNA, *Proc. Natl. Acad. Sci. USA* **48**:532.

Chan, V. L., 1975, On the role of V gene in ultraviolet-induced enhancement of recombination among T4 phages, *Virology* **65**:266.

Chiang, T., and Harm, W., 1974, Evidence against phenotypic mixing between bacteriophage T4 wild type and T4v$^-$, *J. Virol.* **14**:592.

Clark, A. J., and Margulies, A. D., 1965, Isolation and characterization of recombination-deficient mutants of *Escherichia coli* K-12, *Proc. Natl. Acad. Sci. USA* **53**:451.

Cordone, L., Sperandeo-Mineo, R. M., and Mannino, S., 1975, UV-induced enhancement of recombination among lambda bacteriophages: Relation with replication of irradiated DNA, *Nucleic Acid Res.* **2**:1129.

Crick, F. H. C., Barnett, L., Brenner, S., and Watts-Tobin, R. J., 1961, General nature of the genetic code for proteins, *Nature* (*London*) **192**:1227.

Cunningham, R. P., and Berger, H., 1977, Mutations affecting genetic recombination in bacteriophage T4D. I. Pathway analsyis, *Virology* **80**:67.

Cunningham, R. P., Shibata, T., DasGupta, C., and Radding, C. M., 1979, Single strands induce *recA* protein to unwind duplex DNA for homologous pairing, *Nature* (*London*) **281**:191.

Das, A., Court, D., and Adhya, S., 1976, Isolation and characterization of conditional lethal mutants of *Escherichia coli* defective in transcription termination factor *rho*, *Proc. Natl. Acad. Sci. USA* **73**:1959.

DasGupta, C. K., and Poddar, R. K., 1975, Ultraviolet reactivation of the single stranded DNA phage ϕX174, *Mol. Gen. Genet.* **139**:77.

David, C. N., 1964, UV inactivation and thymine dimerization in bacteriophage ϕX, *Z. Vererbungsl.* **95**:318.

Davis, K. J., and Symonds, N., 1974, The pathway of recombination in phage T4, *Mol. Gen. Genet.* **132**:173.

Day, R. S., III, 1977, UV-induced alleviation of K-specific restriction of bacteriophage λ, *J. Virol.* **21**:1249.

Day, R. S., and Muel, B., 1974, Ultraviolet inactivation of the ability of *E. coli* to support the growth of phage T7: An action spectrum, *Photochem. Photobiol.* **20**:95.

Defais, M., Fauquet, P., Radman, M., and Errera, M., 1971, Ultraviolet reactivation and ultraviolet mutagenesis λ in different genetic systems, *Virology* **43**:495.

Defais, M., Caillet-Fauquet, P., Fox, M. S., and Radman, M., 1976, Induction kinetics of mutagenic DNA repair activity in *E. coli* following ultraviolet irradiation, *Mol. Gen. Genet.* **148**:125.

Delihas, N., 1961, The ability of irradiated bacteriophage T2 to initiate the synthesis of deoxycytidylate hydroxymethylase in *Escherichia coli*, *Virology* **13**:242.

Delius, H., Howe, C., and Kozinski, A. W., 1971, Structure of the replicating DNA from bacteriophage T4, *Proc. Natl. Acad. Sci. USA* **68**:3049.

DeLucia, P., and Cairns, J., 1969, Isolation of an *E. coli* strain with a mutation affecting DNA polymerase, *Nature* (*London*) **224**:1164.

Demple, B., and Linn, S., 1980, DNA *N*-glycosylases and UV repair, *Nature* **287**:203.

Denhardt, D. T., and Sinsheimer, R. L., 1965, The process of infection with bacteriophage ϕX174. VI. Inactivation of infected complexes by ultraviolet irradiation, *J. Mol. Biol.* **12**:674.

Devoret, R., and George, J., 1967, Induction indirect du prophage λ par le rayonnement ultraviolet, *Mutat. Res.* **4**:713.

Dewey, M. J., and Frankel, F. R., 1975, Two suppressor loci for gene 49 mutations of bacteriophage T4. I. Genetic properties and DNA synthesis, *Virology* **68**:387.

Dirksen, M.-L., Wiberg, J. S., Koerner, J. F., and Buchanan, J. M., 1960, Effect of ultraviolet irradiation of bacteriophage T2 on enzyme synthesis in host cells, *Proc. Natl. Acad. Sci. USA* **46**:1425.

Doermann, A. H., 1961, The analysis of ultraviolet lesions in bacteriophage T4 by cross reactivation, *J. Cell. Comp. Physiol. Suppl.* **58**(1):79.

Doermann, A. H., Chase, M., and Stahl, F. W., 1955, Genetic recombination and replication in bacteriophage, *J. Cell. Comp. Physiol. Suppl.* **45**(2):51.

Donch, J., Greenberg, J., and Green, M. H. L., 1970, Repression of induction by UV of λ phage by exrA mutations in *Escherichia coli*, *Genet. Res.* **15**:87.

Donch, J. J., Green, M. H. L., and Greenberg, J., 1971, Conditional induction of λ prophage in exrA mutants of *Escherichia coli*, *Genet. Res.* **17**:161.

Drake, J. W., 1963, Properties of ultraviolet-induced rII mutants of bacteriophage T4, *J. Mol. Biol.* **6**:268.

Drake, J. W., 1966*a*, Ultraviolet mutagenesis in bacteriophage T4. I. Irradiation of extracellular phage particles, *J. Bacteriol.* **91**:1775.

Drake, J. W., 1966*b*, Photoreversal of ultraviolet mutagenesis in phage T4, *J. Bacteriol.* **92**:144.

Drake, J. W., 1973, The genetic control of spontaneous and induced mutation rates in bacteriophage T4, *Genetics* (*Suppl.*) **73**:45.

Duckworth, D. H., and Bessman, M., 1967, The enzymology of virus-infected bacteria X. A biochemical-genetic study of the deoxynucleotide kinase induced by wild type and amber mutants of phage T4, *J. Biol. Chem.* **242**:2877.

Dulbecco, R., 1949, Reactivation of ultra-violet-inactivated bacteriophage by visible light, *Nature* (*London*) **163**:949.

Dulbecco, R., 1950, Experiments on photoreactivation of bacteriophages inactivated with ultraviolet radiation, *J. Bacteriol.* **59**:329.

Ebisuzaki, K., 1966, Ultraviolet sensitivity and functional capacity in bacteriophage T4, *J. Mol. Biol.* **20**:545.

Ebisuzaki, K., 1969, Role of polynucleotide ligase and DNA polymerase in the repair of DNA damage, *J. Mol. Biol.* **42**:375.

Echols, H., and Murialdo, H., 1978, Genetic map of bacteriophage lambda, *Microbiol. Rev.* **42**:577.

Eisberg, R. M., 1961, *Fundamentals of Modern Physics*, John Wiley and Sons, New York.

Eitner, G., 1977, The influence of prophage λ on the mutagenic response of *Escherichia coli* to ultraviolet irradiation, *Mutat. Res.* **44**:299.

Ellison, S. A., Feiner, R. R., and Hill, R. F., 1960, A host effect on bacteriophage survival after ultraviolet irradiation, *Virology* **11**:294.

Emmerson, P. T., 1968, Recombination deficient mutants of *Escherichia coli* K12 that map between thyA and argA, *Genetics* **60**:19.

Emmerson, P. T., and Howard-Flanders, P., 1967, Cotransduction with thy of a gene required for genetic recombination in *Escherichia coli*, *J. Bacteriol.* **93**:1729.

Emmerson, P. T., and West, S. C., 1977, Identification of protein X of *Escherichia coli* as the recA$^+$/tif$^+$ gene product, *Mol. Gen. Genet.* **155**:77.

Epstein, R. H., 1958, A study of multiplicity-reactivation in the bacteriophage T4, *Virology* **6**:382.

Fisher, D. B., and Pardee, A. B., 1968, The Luria-Latarjet effect studied by phage T4-lysozyme production, *Virology* **34**:91.

Flaks, J. G., and Cohen, S. S., 1959, Virus-induced acquisition of metabolic function. I. Enzymatic formation of 5-hydroxymethyldeoxycytidylate, *J. Biol. Chem.* **234**: 1501.

Folsome, C. E., and Levin, D., 1961, Detecting reversion in T4rII bacteriophage to r$^+$ induced by ultra-violet irradiation, *Nature* (*London*) **192**:1306.

Fornili, S. L., Cordone, L., and Radman, M., 1971, Phenomenological theory of repair kinetics of ultraviolet irradiated lambda phage, *Virology* **43**:422.

Francke, B., and Ray, D. S., 1971, Formation of the parental replicative form DNA of bacteriophage ϕX174 and initial events in its replication, *J. Mol. Biol.* **61**:565.

Francke, B., and Ray, D. S., 1972, Ultraviolet- induced cross-links in the deoxyribonucleic acid of single-stranded deoxyribonucleic acid viruses as a probe of deoxyribonucleic acid packaging, *J. Virol.* **9**:1027.

Freese, E., 1959*a*, The specific mutagenic effect of base analogues on phage T4, *J. Mol. Biol.* **1**:87.

Freese, E., 1959*b*, The difference between spontaneous and base-analogue induced mutations of phage T4, *Proc. Natl. Acad. Sci. USA* **45**:622.

Freese, E., Bautz, E., and Bautz Freese, E., 1961, The chemical and mutagenic specificity of hydroxylamine, *Proc. Natl. Acad. Sci. USA* **47**:845.

Friedberg, E. C., 1972, Studies on the substrate specificity of the T4 excision repair endonuclease, *Mutat. Res.* **15**:113.

Friedberg, E. C,. 1975, DNA repair of ultraviolet-irradiated bacteriophage T4, *Photochem. Photobiol.* **21**:277.

Friedberg, E. C., and King, J. J., 1969, Endonucleolytic cleavage of UV-irradiated DNA controlled by the V+ gene in phage T4, *Biochem. Biophys. Res. Commun.* **37**:646.

Friedberg, E. C., and King, J. J., 1971, Dark repair of ultraviolet-irradiated deoxyribonucleic acid by bacteriophage T4: Purification and characterization of a dimer-specific phage-induced endonuclease, *J. Bacteriol.* **106**:500.

Fuerst, C. R., and Siminovitch, L., 1965, Characterization of an unusual defective lysogenic strain of *Escherichia coli* K12 (λ), *Virology* **27**:449.

Geissler, E., 1970, Differences in host controlled reactivation of lambdoid and T phages, *Mol. Gen. Genet.* **109**:257.

Gellert, M., 1967, Formation of covalent circles of λ DNA by *E. coli* extracts, *Proc. Natl. Acad. Sci. USA* **57**:148.

Gellert, M., Mizuuchi, K., O'Dea, M. H., Itoh, T., and Tomizawa, J. I., 1977, Naladixic acid resistance: A second character involved in DNA gyrase activity, *Proc. Natl. Acad. Sci. USA* **74**:4772.

George, D., and Rosenberg, D., 1972, Host cell deoxyribonucleic acid polymerase I and the support of T4 bacteriophage growth, *J. Bacteriol.* **112:**1017.

George, J., and Devoret, R., 1971, Conjugal transfer of UV-damaged F-prime sex factors and indirect induction of prophage λ, *Mol. Gen. Genet.* **111:**103.

George, J., Devoret, R., and Radman, M., 1974, Indirect ultraviolet reactivation of phage λ, *Proc. Natl. Acad. Sci. USA* **71:**144.

Godson, G. N., 1971, Characterization and synthesis of φX174 proteins in ultraviolet-irradiated and unirradiated cells, *J. Mol. Biol.* **57:**541.

Goldmark, P. J., and Linn, S., 1970, An endonuclease activity from *Escherichia coli* absent from certain rec$^-$ strains, *Proc. Natl. Acad. Sci. USA* **67:**434.

Goldthwait, D., and Jacob, F., 1964, Sur le mechanisme de l'induction du developpement du prophage chez les bacteries lysogenes, *C. R. Acad. Sci.* **259:**661.

Gottesman, M. M., Gottesman, M. E., Gottesman, S., and Gellert, M., 1974, Characterization of bacteriophage λ reverse as an *Escherichia coli* phage carrying a unique set of host-derived recombination functions, *J. Mol. Biol.* **88:**471.

Green, R. R., and Drake, J. W., 1974, Misrepair mutagenesis in bacteriophage T4, *Genetics* **78:**81.

Gross, J., and Gross, M., 1969, Genetic analysis of an *E. coli* strain with a mutation affecting DNA polymerase, *Nature* (*London*) **224:**116.

Gudas, L. J., 1976, The induction of protein X in DNA repair and cell division mutants of *Escherichia coli*, *J. Mol. Biol.* **104:**567.

Gudas, L. J., and Mount, D. W., 1977, Identification of the recA (tif) gene product of *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* **74:**5280.

Gudas, L. J., and Mount, D. W., 1978, An *Escherichia coli* mutant with an altered inducible rec$^+$/lex$^+$ dependent DNA repair pathway, in: *DNA Repair Mechanisms* (P. C. Hanawalt, E. C. Friedberg, and C. F. Fox, eds.), pp. 361–365, Academic Press, New York.

Gudas, L. J., and Pardee, A. B., 1975, Model for regulation of *Escherichia coli* DNA repair functions, *Proc. Natl. Acad. Sci. USA* **72:**2330.

Gudas, L. J., and Pardee, A. B., 1976, DNA synthesis inhibition and the induction of protein X in *Escherichia coli*, *J. Mol. Biol.* **101:**459.

Hamlett, N. V., and Berger, H., 1975, Mutations altering genetic recombination and repair of DNA in bacteriophage T4, *Virology* **63:**539.

Hanawalt, P. C., Copper, P. K., Ganesan, A. K., and Smith, C. A., 1979, DNA repair in bacteria and mammalian cells, *Annu. Rev. Biochem.* **48:**783.

Hariharan, P. V., and Cerutti, P. A., 1977, Formation of products of the 5,6 dihydroxydihydrothymine type by UV light in Hela cells, *Biochemistry* **16:**219.

Harm, W., 1956, On the mechanism of multiplicity reactivation in bacteriophage, *Virology* **2:**559.

Harm, W., 1961, Gene-controlled reactivation of ultraviolet-inactivated bacteriophage, *J. Cell. Comp. Physiol. Suppl.* **58:**(1):69.

Harm, W., 1963*a*, On the relationship between host-cell reactivation and UV-reactivation in UV-inactivated phages, *Z. Vererbungsl.* **94:**67.

Harm, W., 1963*b*, Mutants of phage T4 with increased sensitivity to ultraviolet, *Virology* **19:**66.

Harm, W., 1964, On the control of UV-sensitivity of phage T4 by the gene x, *Mutat. Res.* **1:**344.

Harm, W., 1965, Host-cell reactivation of non-lethal ultraviolet effects, *Photochem. Photobiol.* **4:**575.

Harm, W., 1968, Recovery of UV-inactivated *E. coli* cells by the *v*-gene action of phage T4, *Mutat. Res.* **6**:175.

Harm, W., 1969, Biological determination of the germicidal activity of sunlight, *Radiat. Res.* **40**:63.

Harm, W., 1973, T4-mediated *v*-gene reactivation of UV-irradiated phage T1, and its comparison with host cell reactivation, *Mutat. Res.* **17**:163.

Hart, M. G. R., and Ellison, J., 1970, Ultraviolet reactivation in bacteriophage lambda, *J. Gen. Virol.* **8**:197.

Haseltine, W. A., Gordon, L. K., Lindan, C. P., Grafstrom, R. H., Shaper, N. L., and Grossman, L., 1980, Cleavage of pyrimidine dimers in specific DNA sequences by a primidine dimer DNA-glycosylase of M. luteus, *Nature* **285**:634.

Hatchard, C. G., and Parker, C. A., 1956, A new sensitive chemical actinometer. II. Potassium ferrioxalate as a standard chemical actinometer, *Proc. R. Soc. (London) Ser. A* **235**:518.

Hays, J. B., and Boehmer, S., 1978, Antagonists of DNA gyrase inhibit repair and recombination of UV-irradiated phage λ, *Proc. Natl. Acad. Sci. USA* **75**:4125.

Hertman, I., and Luria, S. E., 1967, Transduction studies on the role of a rec$^+$ gene in the UV induction of prophage λ, *J. Mol. Biol.* **23**:117.

Hill, R. F., 1958, A radiation-sensitive mutant of *Escherichia coli*, *Biochim. Biophys. Acta* **30**:636.

Hill, R. F., and Feiner, R. R., 1963, Further studies of ultraviolet-sensitive mutants of *Escherichia coli* strain *J. Gen. Microbiol.* **35**:105.

Hill, R. F., and Simson, E., 1961, A study of radiosensitive and radioresistant mutants of *Escherichia coli* strain B, *J. Gen. Microbiol.* **24**:1.

Horii, Z. I., and Clark, A. J., 1973, Genetic analysis of the *rec* F pathway to genetic recombination in *Escherichia coli* K12: Isolation and characterization of mutants, *J. Mol. Biol.* **80**:327.

Horiuchi, T., and Nagata, T., 1973, Mutations affecting growth of the *Escherichia coli* cell under a condition of DNA polymerase I-deficiency, *Mol. Gen. Genet.* **123**:89.

Howard-Flanders, P., and Boyce, R. P., 1966, DNA repair and genetic recombination: Studies of mutants of *Escherichia coli* defective in these processes, *Radiat. Res. Suppl.* **6**:156.

Howard-Flanders, P., and Theriot, L., 1962, A method for selecting radiation-sensitive mutants of *Escherichia coli*, *Genetics* **47**:1219.

Howard-Flanders, P., and Theriot, L., 1966, Mutants of *Escherichia coli* K-12 defective in DNA repair and in genetic recombination, *Genetics* **53**:1137.

Howard-Flanders, P., Boyce, R. P., Simson, E., and Theriot, L., 1962, A genetic locus in *E. coli* K12 that controls the reactivation of UV photoproducts associated with thymine in DNA, *Proc. Natl. Acad. Sci. USA* **48**:2109.

Howard-Flanders, P., Boyce, R. P., and Theriot, L., 1966, Three loci in *Escherichia coli* K-12 that control the excision of the pyrimidine dimers and certain other mutagen products from DNA, *Genetics* **53**:1119.

Howe, C. C., Buckley, P. J., Carlson, K. M., and Kozinski, A. W., 1973, Multiple and specific initiation of T4 replication, *J. Virol.* **12**:139.

Huskey, R. J., 1969, Multiplicity reactivation as a test for recombination function, *Science* **164**:319.

Ichikawa-Ryo, H., and Kondo, S., 1975, Indirect mutagenesis in phage lambda by ultraviolet preirradiation of host bacteria, *J. Mol. Biol.* **97**:77.

Inouye, M., 1971, Pleiotropic effect of the *rec*A gene of *Escherichia coli: Uncoupling of cell division from deoxribonucleic acid replication*, *J. Bacteriol.* **106**:539.

Inouye, M., and Guthrie, J. P., 1969, A mutation which changes a membrane protein of *E. coli*, *Proc. Natl. Acad. Sci. USA* **64**:957.

Inouye, M., and Pardee, A. B., 1970, Changes of membrane proteins and their relation to deoxyribonucleic acid synthesis and cell division of *Escherichia coli*, *J. Biol. Chem.* **245**:5813.

Ito, M., and Sekiguchi, M., 1976, Repair of DNA damaged by 4-nitroquinoline-1-oxide: A comparison of *Escherichia coli* and bacteriophage T4 systems, *Jpn. J. Genet.* **51**:129.

Jacob, F., and Campbell, A., 1959, Sur le systeme de repression assurant l'immunite chez les bacteries lysogenes, *C. R. Acad. Sci.* **248**:3219.

Jacob, F., and Wollman, E. L., 1953, Induction of phage development in lysogenic bacteria, *Cold Spring Harbor Symp. Quant. Biol.* **18**:101.

Jacob, F., and Wollman, E.-L., 1955, Etude genetique, d'un bacteriophage tempere d'*Escherichia coli*. III. Effet du rayonnement ultraviolet sur la recombinaison genetique, *Ann. Inst. Pasteur* (*Paris*) **88**:724.

Jagger, J., 1961, A small and inexpensive ultraviolet dose-rate meter useful in biological experiments, *Radiat. Res.* **14**:394.

Jagger, J., 1967, *Introduction to Research in Ultraviolet Photobiology*, Prentice-Hall, Englewood Cliffs, N.J.

Jansz, H. S., Pouwels, P. H., and Van Rotterdam, C., 1963, Sensitivity to ultraviolet light of single- and double-stranded DNA, *Biochim. Biophys. Acta* **76**:655.

Johns, H. E., 1969, Photochemical reactions in nucleic acids, in: *Methods in Enzymology*, Vol. XVI (K. Kustin, ed.), pp. 253–316, Academic Press, New York.

Kato, T., and Shinoura, Y., 1977, Isolation and characterization of mutants of *Escherichia coli* deficient in induction of mutations by ultraviolet light, *Mol. Gen. Genet.* **156**:121.

Kellenberger, G., and Weigle, J. J., 1958, Etude au moyen des rayons ultraviolets de l'interaction entre bacteriophage tempere et bacterie hote, *Biochim. Biophys. Acta* **30**:112.

Kerr, T. L., and Hart, M. G. R., 1972, Effects of the *rec* and *exr* mutations of *Escherichia coli* on UV reactivation of bacteriophage lambda damaged by different agents, *Mutat. Res.* **15**:247.

Kerr, T. L., and Hart, M. G. R., 1973, Effects of the *recA*, *lex*, and *exrA* mutations on the survival of damaged and P1 phages in lysogenic and non-lysogenic strains of Escherichia coli, *Mutat. Res.* **18**:113.

Kirby, E. P., Jacob, F., and Goldthwait, D. A., 1967, Prophage induction and filament formation in a mutant strain of *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* **58**:1903.

Klein, A., and Niebch, U., 1971, Host cell reactivation in strains of E. coli lacking DNA polymerase activity *in vitro*, *Nature* (*London*) *New Biol.* **229**:82.

Kneser, H., 1966, Absence of K-reactivation in a recombination-deficient mutant of *E. coli*, *Biochem. Biophys. Res. Commun.* **22**:383.

Kneser, H., 1968, Relationship between K-reactivation and UV reactivation of bacteriophage, *Virology* **36**:303.

Kobayashi, I., and Ikeda, H., 1977, Formation of recombinant DNA of bacteriophage λ by *recA* function of *Escherichia coli* without duplication, transcription, translation, and maturation, *Mol. Gen. Genet.* **153**:237.

Kobayashi, I., and Ikeda, H., 1978, On the role of *recA* gene product in genetic recombination: An analysis by *in vitro* packaging of recombinant DNA molecules formed in the absence of protein synthesis, *Mol. Gen. Genet*, **166**:25.

Kozinski, A. W., and Doermann, A. H., 1975, Repetitive DNA replication of the incomplete genomes of phage T4 petite particles, *Proc. Natl. Acad. Sci. USA* **72**:1734.

Kozinski, A. W., Doermann, A. H., and Kozinski, P. B., 1976, Absence of interparental recombination in multiplicity reconstitution from incomplete bacteriophage T4 genomes, *J. Virol.* **18**:873.

Krieg, D. R., 1959*a*, A study of gene action in ultraviolet-irradiated T4, *Virology* **8**:80.

Krieg, D., 1959*b*, Induced reversion of T4*rII* mutants by ultraviolet irradiation of extracellular phage, *Virology* **9**:215.

Krisch, H. M., and Van Houwe, G., 1976, Stimulation of the synthesis of bacteriophage T4 gene 32 protein by ultraviolet light irradiation, *J. Mol. Biol.* **108**:67.

Krisch, H. M., Bolle, A., and Epstein, R. H., 1974, Regulation of the synthesis of bacteriophage T4 gene 32 protein, *J. Mol. Biol.* **88**:89.

Krogdahl, W. S., 1962, *The Astronomical Universe*, Macmillan, New York.

Kushner, S. R., Nagaishi, H., Templin, A., and Clark, A. J., 1971, Genetic recombination in *Escherichia coli:* The role of exonuclease I, *Proc. Natl. Acad. Sci. USA* **68**:824.

Kushner, S. R., Nagaishi, H., and Clark, A. J., 1972, Indirect suppression of *recB* and *recC* mutations by exonuclease I deficiency, *Proc. Natl. Acad. Sci. USA* **69**:1366.

Kushner, S. R., Nagaishi, H., and Clark, A. J., 1974, Isolation of exonuclease. VIII. The enzyme associated with the *sbcA* indirect suppressor, *Proc. Natl. Acad. Sci. USA* **71**:3593.

Kushner, S. R., Shepard, J., Edwards, G., and Maples, V. F., 1978, *uvrD*, *uvrE*, and *recL* represent a single gene, in: *DNA Repair Mechanisms* (P. C. Hanawalt, E. C. Friedberg, and C. F. Fox, eds.), pp. 251–254, Academic Press, New York.

Latarjet, R., 1949, Mutation induite chez un virus par irradiation ultraviolette de cellules infectees, *C. R. Acad. Sci. (Paris)* **228**:1354.

Latarjet, R., 1954, Mutations induites chez un bacteriophage, *Acta Unio Int. Contra Cancrum* **10**:136.

Latarjet, R., and Wahl, R., 1945, Precisions sur l'inactivation des bacteriophages par les rayons ultraviolets, *Ann. Inst. Pasteur (Paris)* **71**:336.

Latarjet, R., Morenne, P., and Berger, R., 1953, Un appareil simple pour le dosage des rayonnements ultraviolets emis par les lampes germicides, *Ann. Inst. Pasteur (Paris)* **85**:174.

Lederberg, E. M., 1951, Lysogenicity in *E. coli* K-12, *Genetics* **36**:560.

Lieb, M., 1964, Ultraviolet sensitivity of *Escherichia coli* containing heat-inducible λ prophages, *Science* **145**:175.

Lin, P. F., and Howard-Flanders, P., 1976, Genetic exchanges caused by ultraviolet photoproducts in phage λ DNA molecules: The role of DNA replication, *Mol. Gen. Genet.* **146**:107.

Little, J. W., and Hanawalt, P. C., 1977, Induction of protein X in *Escherichia coli*, *Mol. Gen. Genet.* **150**:237.

Little, J. W., and Kleid, D. G., 1977, *Escherichia coli* protein X is the *recA* gene product, *J. Biol. Chem.* **252**:6251.

Low, B., 1968, Formation of merodiploids in matings with a class of *rec*$^-$ recipient strains of *Escherichia coli* K12, *Proc. Natl. Acad. Sci. USA* **60**:160.

Luria, S. E., 1944, A growth delaying effect of ultraviolet radiation on bacterial viruses, *Proc. Natl. Acad. Sci. USA* **30**:393.

Luria, S. E., 1947, Reactivation of irradiated bacteriophage by transfer of self-reproducing units, *Proc. Natl. Acad. Sci. USA* **33**:253.

Luria, S. E., 1949, Type hybrid bacteriophages, *Rec. Genet. Soc. Am.* **18**:102.

Luria, S. E., and Dulbecco, R., 1949, Genetic recombinations leading to production of active bacteriophage from ultraviolet inactivated particles, *Genetics* **34**:93.

Luria, S. E., and Latarjet, R., 1947, Ultraviolet irradiation of bacteriophage during intracellular growth, *J. Bacteriol.* **53**:149.

Lwoff, A., 1951, Conditions de l'efficacite inductice du rayonnement ultraviolet chez une bacterie lysogene, *Ann. Inst. Pasteur* (*Paris*) **81**:370.

Lwoff, A., 1953, Lysogeny, *Bacteriol. Rev.* **17**:269.

Lwoff, A., Siminovitch, L., and Kjelgaard, N., 1950, Induction de la production de bacteriophages chez une bacterie lysogene, *Ann. Inst. Pasteur* (*Paris*) **79**:815.

Lydersen, B. K., and Pollard, E. C., 1977, Ultraviolet induction of prophage lambda during inhibition of deoxyribonucleic acid synthesis by hydroxyurea, *J. Bacteriol.* **129**:698.

Lytle, C. D., Goddard, J. G., and Buchta, F. L., 1978, Protease inhibitors prevent UV-enhanced virus reactivation in *E. coli* but not in monkey kidney cells, in: *DNA Repair Mechanisms* (P. C. Hanawalt, E. C. Friedberg, and C. F. Fox, eds.), pp. 559–562, Academic Press, New York.

Makino, F., Tanooka, H., and Sekiguchi, M., 1977, Action of T4 endonuclease V on DNA containing various photoproducts, *J. Biochem.* **82**:1567.

Mandel, M., and Higa, A., 1970, Calcium-dependent bacteriophage DNA infection, *J. Mol. Biol.* **53**:159.

Marinus, M. C., and Morris, N. R., 1974, Biological function for 6-methyladenine residues in the DNA of *Escherichia coli* K12, *J. Mol. Biol.* **85**:309.

Masamune, Y., 1976, Effect of ultraviolet irradiation of bacteriophage f1 DNA on its conversion to replicative form by extracts of *Escherichia coli*, *Mol. Gen. Genet.* **149**:335.

Matsubara, K., Shimada, K., and Takagi, Y., 1967, Replication process of single-stranded DNA of bacteriophage ϕX174: Production of replicative form in *Escherichia coli* cells irradiated by UV light, *J. Mol. Biol.* **29**:297.

Mattern, I. E., and Houtman, P. C., 1974, Properties of *uvrE* mutants of *Escherichia coli* K12. II. Construction and properties of *pol* and *rec* derivatives, *Mutat. Res.* **25**:287.

Mattern, I. E., Van Winden, M. P., and Rorsch, A., 1965, The range of action of genes controlling radiation sensitivity in *Escherichia coli*, *Mutat. Res.* **2**:111.

Mattern, I., Zwenk, H., and Rorsch, A., 1966, The genetic constitution of the radiation-sensitive mutant *Escherichia coli* Bs-1, *Mutat. Res.* **3**:374.

Maynard-Smith, S., and Symonds, N., 1973*a*, Involvement of bacteriophage T4 genes in radiation repair, *J. Mol. Biol.* **74**:33.

Maynard-Smith, S., and Symonds, N., 1973*b*, The unexpected location of a gene conferring abnormal radiation sensitivity on phage T4, *Nature* (*London*) **241**:395.

Maynard-Smith, S., Symonds, N., and White, P., 1970, The Kornberg polymerase and the repair of irradiated T4 bacteriophage, *J. Mol. Biol.* **54**:391.

McCarthy, D., 1979, Gyrase-dependent initiation of bacteriophage T4 DNA replication: Interactions of *Escherichia coli* gyrase with novobiocin, coumermycin, and phage DNA-delay gene products, *J. Mol. Biol.* **127**:265.

McEntee, K., 1977, Protein X is the product of the *recA* gene of *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* **74**:5275.

McEntee, K., Hesse, J. E., and Epstein, W., 1976, Identification and radiochemical purification of the *recA* protein of *Escherichia coli* K12, *Proc. Natl. Acad. Sci. USA* **73**:3979.

Meistrich, M. L., 1972, Contribution of thymine dimers to the ultraviolet light inactivation of mutants of bacteriophage T4, *J. Mol. Biol.* **66**:97.

Meistrich, M. L., and Drake, J. W., 1972, Mutagenic effects of thymine dimers in bacteriophage T4, *J. Mol. Biol.* **66**:107.

Meistrich, M. L., and Lamola, A. A., 1972, Triplet-state sensitization of thymine photodimerization in bacteriophage T4, *J. Mol. Biol.* **66**:83.

Metzger, K., 1964, On the darkreactivation mechanism in ultraviolet irradiated bacteria, *Biochem. Biophys. Res. Commun.* **15**:101.

Meyn, M. S., Rossman, T., and Troll, W., 1977, A protease inhibitor blocks SOS functions in *Escherichia coli:* Antipain prevents λ repressor inactivation, ultraviolet mutagenesis and filamentous growth, *Proc. Natl. Acad. Sci. USA* **74**:1152.

Meyn, M. S., Rossman, T., Gottlieb, P., and Troll, W., 1978, The role of proteases in SOS regulation, in *DNA Repair Mechanisms* (P. C. Hanawalt, E. C. Friedberg, and C. F. Fox, eds.), pp. 379–382, Academic Press, New York.

Minton, K., Durphy, M., Taylor, R., and Friedberg, E. C., 1975, The ultraviolet endonuclease of bacteriophage T4: further characterization, *J. Biol. Chem.* **250**:2823.

Miura, A., 1974, Ultraviolet-induced mutation of bacteriophage λ:λ induction by conjugation with ultraviolet irradiated donor cells, *Mol. Gen. Genet.* **134**:21.

Miura, A., and Tomizawa, J. -I., 1968, Studies on radiation-sensitive mutants of *E. coli.* III. Participation of the *rec* system in induction of mutation by ultraviolet-irradiation, *Mol. Gen. Genet.* **103**:1.

Monk, M., Peacey, M., and Gross, J. D., 1971, Repair of damage induced by ultraviolet light in DNA polymerase defective *E. coli* cells, *J. Mol. Biol.* **58**:623.

Moreau, P., Bailone, A., and Devoret, R., 1976, Prophage induction in *Escherichia coli* K12 *envA uvrB:* A highly sensitive test for potential carcinogens, *Proc. Natl. Acad. Sci. USA* **73**:3700.

Mortelmans, K., and Friedberg, E. C., 1972, Deoxyribonucleic acid repair in bacteriophage T4: Observations on the role of the *x* and *v* genes and of host factors, *J. Virol.* **10**:730.

Mosig, G., Bowden, D. W., and Bock, S., 1972, *E. coli* DNA polymerase I and other host functions participate in T4 DNA replication and recombination, *Nature (London) New Biol.* **240**:12.

Mount, D. W., 1977, A mutant of *Escherichia coli* showing constitutive expression of the lysogenic induction and error-prone DNA repair pathways, *Proc. Natl. Acad. Sci. USA* **74**:300.

Mount, D. W., Low, R. B., and Edmiston, S. J., 1972, Dominant mutations (*lex*), in *Escherichia coli* K-12 which affect radiation sensitivity and frequency of ultraviolet light-induced mutations, *J. Bacteriol.* **112**:886.

Mount, D. W., Walker, A. C., and Kusel, C., 1973, Suppression of *lex* mutations affecting deoxyribonucleic acid repair in *Escherichia coli* K-12 by closely linked thermosensitive mutations, *J. Bacteriol.* **116**:950.

Mount, D. .W, Kosel, C. K., and Walker, A., 1976, Inducible, error-free DNA repair in *ts1 recA* mutants of *E. coli*, *Mol. Gen. Genet.* **146**:37.

Munro, J. L., and Wiberg, J. S., 1968, Evidence that gene 56 of bacteriophage T4 is a structural gene for deoxycytidine triphosphatase, *Virology* **38**:442.

Nobrega, F. G., Rola, F. H., Pasetto-Nobrega, M., and Oishi, M., 1972, Adenosine triphosphatase associated with adenosine triphosphate-dependent deoxyribonuclease, *Proc. Natl. Acad. Sci. USA* **69**:15.

Ogawa, H., 1970, Genetic location of *uvrD* and *pol* genes of *E. coli*, *Mol. Gen. Genet.* **108**:378.

Ogawa, H., and Tomizawa, J.-I., 1973, Ultraviolet reactivation of lambda phage: Assay of infectivity of DNA molecules by spheroplast transfection, *J. Mol. Biol.* **73**:397.

Ogawa, H., Shimada, K., and Tomizawa, J.-I., 1968, Studies on radiation-sensitive mutants of *E. coli*. I. Mutants defective in the repair synthesis, *Mol. Gen. Genet.* **101**:227.

Oishi, M., 1969, An ATP-dependent deoxyribonuclease from *Escherichia coli* with a possible role in recombination, *Proc. Natl. Acad. Sci. USA* **64**:1292.

Ono, J., and Shimazu, Y., 1966, Ultraviolet reactivation of a bacteriophage containing a single-stranded deoxyribonucleic acid as a genetic element, *Virology* **29**:295.

Orgel, A., and Brenner, S., 1961, Mutagenesis of bacteriophage T4 by acridines, *J. Mol. Biol.* **3**:762.

Patrick, M. H., and Rahn, R. O., 1976, Photochemistry of DNA and polynucleotides: Photoproducts, in: *Photochemistry and Photobiology of Nucleic Acids*, Vol. II, (S. Y. Wang, ed.), pp. 35–95, Academic Press, New York.

Pauling, C., and Hamm, L., 1968, Properties of a temperature-sensitive radiation-sensitive mutant of *Escherichia coli*, *Proc. Natl. Acad. Sci. USA* **60**:1495.

Pauling, C., and Hamm, L., 1969*a*, Properties of a temperature-sensitive radiation-sensitive mutant of *Escherichia coli*. II. DNA replication, *Proc. Natl. Acad. Sci. USA* **64**:1969.

Pauling, C., and Hamm, L., 1969b, Properties of a DNA ligase mutant of *Escherichia coli*. II. Intermediates in DNA replication, Biochem. Biophys. Res. Commun. **37**:1015.

Pawl, G., Taylor, R., Minton, K., and Friedberg, E. C., 1976, Enzymes involved in thymine dimer excision in bacteriophage T4-infected *Escherichia coli*, *J. Mol. Biol.* **108**:99.

Pettijohn, D., and Hanawalt, P., 1964, Evidence for repair-replication of ultraviolet-damaged DNA in bacteria, *J. Mol. Biol.* **9**:305.

Poddar, R. K., and Sinsheimer, R. L., 1971, Nature of the complementary strands synthesized *in vitro* upon the single-stranded circular DNA of bacteriophage ϕX174 after ultraviolet irradiation, *Biophys. J.* **11**:355.

Prakash, R. K., Netrawali, M. S., Pradhan, D. S., and Sreenivasan, A., 1975, Ineffectivenss of rifampicin in inhibiting RNA synthesis in *Escherichia coli* and T4-infected *Escherichia coli* cells after exposure to ultraviolet radiation, *Biochim. Biophys. Acata* **383**:435.

Premier, M. M., and Chan, V. L., 1978, The effects of virus and host genes on recombination among ultraviolet-irradiated bacteriophage T4, *Virology* **88**:338.

Radany, E. H., and Friedberg, E. C., 1980, A pyrimidine dimer-DNA glycosylase activity associated with the *v* gene product of bacteriophage T4, *Nature* **286**:182.

Radman, M., 1974, Phenomenology of an inducible mutagenic DNA repair pathway in *Escherichia coli:* SOS repair hypothesis, in: *Molecular and Environmental Aspects of Mutagenesis* (L. Prakash, F., Sherman, M. W. Miller, C. W. Lawrence, and H. W. Taylor, eds.), pp. 128–142, C C Thomas, Springfield, Ill.

Radman, M., and Devoret, R., 1971, UV-reactivation of bacteriophage λ in excision repair-deficient hosts: Independence of red functions and attachment regions, *Virology* **43**:504.

Radman, M., Cordone, L., Krsmanovic-Simic, D., and Errera, M., 1970, Complementary action of recombination and excision in the repair of ultraviolet irradiation damage to DNA, *J. Mol. Biol.* **49**:203.

Radman, M., Villani, G., Boiteux, S., Defais, M., and Caillet-Fauquet, P., 1977, On

the mechanism and genetic control of mutagenesis induced by carcinogenic mutagens, in: *Origins of Human Cancer* (H. H. Hiatt, I. D. Watson, and J. A. Winsten, eds.), pp. 903–922, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

Rahn, R. O., 1979, Nondimer damage in deoxyribonucleic acid caused by ultraviolet irradiation, in: *Photochemical and Photobiological Reviews*, Vol. 4 (K Smith, ed.), pp. 267–330, Plenum Press, New York.

Rahn, R. O., and Patrick, M. H., 1976, Photochemistry of DNA: Secondary structure, photosensitization, base substitution, and exogenous molecules, in: *Photochemistry and Photobiology of Nucleic Acids*, Vol. 2 (S. Y. Wang, ed.), pp. 97–145, Academic Press, New York.

Rahn, R. O., and Sellin, H. G., 1979, Quantum yield of photohydration for dimethyluracil. Concentration and wavelength dependence, *Photochem. Photobiol.* **30**:317.

Ray, U., Bartenstein, L., and Drake, J. W., 1972, Inactivation of bacteriophage T4 by ethyl methanesulfonate: Influence of host and viral genotypes, *J. Virol.* **9**:440.

Rayssiguier, C., and Vigier, P. R., R., 1977, Genetic evidence for the existence of partial replicas of T4 genomes inactivated by irradiation under ultraviolet light, *Virology* **78**:442.

Riazuddin, S., and Grossman, L., 1977*a*, *Micrococcus luteus* correndonucleases. I. Resolution and purification of two endonucleases specific for DNA containing pyrimidine dimers, *J. Biol. Chem.* **252**:6280.

Riazuddin, S., and Grossman, L., 1977*b*, *Micrococcus luteus* correndonucleases. II. Mechanism of action of two endonucleases specific for DNA containing pyrimidine dimers, *J. Biol. Chem.* **252**:6287.

Riazuddin, S., Grossman, L., and Mahler, I., 1977, *Micrococcus luteus* correndonucleases. III. Evidence for involvement in repair *in vivo* of two endonucleases specific for DNA containing pyrimidine dimers, *J. Biol. Chem.* **252**:6294.

Ritchie, D. A., and Symonds, N., 1970, The relation between radiation stability and DNA replication in bacteriophage T4, *J. Gen. Virol.* **8**:121.

Roberts, J. W., and Roberts, C. W., 1975, Proteolytic cleavage of bacteriophage lambda repressor in induction, *Proc. Natl. Acad. Sci. USA* **72**:147.

Roberts, J. W., Roberts, C. W., and Mount, D. W., 1977, Inactivation and proteolytic cleavage of phage λ repressor *in vitro* in an ATP-dependent reaction, *Proc. Natl. Acad. Sci. USA* **74**:2283.

Roberts, J. W., Roberts, C. W., and Craig, N. L., 1978, *Escherichia coli* recA gene product inactivates phage λ repressor, *Proc. Natl. Acad. Sci. USA* **75**:4714.

Rorsch, A., Edelman, A., Van der Kamp, C., and Cohen, J. A., 1962, Phenotypic and genotypic characterization of radiation sensitivity in *Escherichia coli* B, *Biochim. Biophys. Acta* **61**:278.

Rorsch, A., Edelman, A., and Cohen, J. A., 1963, The gene controlled radiation sensitivity in *Escherichia coli*, *Biochim. Biophys. Acta* **68**:263.

Rosenthal, I., and Bercovici, T., 1976, A chemical actinometer for the measurement of UV radiation intensity in the atmosphere, *Atmos. Environ.* **10**:1139.

Rosner, J. L., Kass, L. R., and Yarmolinsky, M., 1968, Parallel behavior of *F* and P1 in causing indirect induction of lysogenic bacteriophage, *Cold Spring Harbor Symp. Quant. Biol.* **33**:785.

Rothman, R. H., 1978, Dimer excision and repair replication patch size in a *recL* 152 mutant of *Escherichia coli* K-12, *J. Bacteriol.* **136**:444.

Rothman, R. H., and Clark, A. J., 1977*a*, Defective excision and postreplication repair

of UV-damaged DNA in a *recL* mutant strain of *E. coli* K-12, *Mol. Gen. Genet.* **155**:267.

Rothman, R. H., and Clark, A. J., 1977*b*, The dependence of postreplication repair on *uvrB* in a *recF* mutant of *Escherichia coli* K12, *Mol. Gen. Genet.* **155**:279.

Rothman, R. H., Margossian, L. J., and Clark, A. J., 1979, W-reactivation of phage λ in *recF*, *recL*, *uvrA*, and *uvrB* mutants, *Mol. Gen. Genet.* **169**:279.

Roulland-Dussoix, D., 1967, Degradation par la cellule hote du DNA du bacteriophage lambda irradie par le rayonnement ultraviolet, *Mutat. Res.* **4**:241.

Runnels, J., and Synder, L., 1978, Isolation of a bacterial host selective for bacteriophage T4 containing cystosine in its DNA, *J. Virol.* **27**:815.

Rupert, C. S., 1975, Enzymatic photoreactivation: Overview, in: *Molecular Mechanisms for Repair of DNA* (P. C. Hanawalt and R. B. Setlow, eds.), pp. 73–87, Plenum Press, New York.

Rupert, C. S., and Harm. W., 1966, Reactivation of biological damage, in: *Advances in Radiation Biology*, Vol. 2 (L. G. Augenstein, R. Mason, and M. Zelle, eds.), pp. 1–81, Academic Press, New York.

Rupp, W. D., and Howard-Flanders, P., 1968, Discontinuities in the DNA synthesized in an excision-defective strain of *Escherichia coli* following ultraviolet irradiation, *J. Mol. Biol.* **31**:29.

Sarasin, A., Goze, A., Devoret, R., and Moule, Y., 1977, Induced reactivation of UV-damaged phage λ in *E. coli* K12 host cells treated with aflatoxin B1, *Mutat. Res.* **42**:205.

Sarthy, P. V., and Meselson, M., 1976, Single burst study of *rec*$^-$ and *red*$^-$ irradiated recombination in bacteriophage lambda, *Proc. Natl. Acad. Sci. USA* **73**:4613.

Sato, K., and Sekiguchi, M., 1976, Studies on temperature-dependent ultraviolet-light-sensitive mutants of bacteriophage T4: The structural gene for T4 endonuclease V, *J. Mol. Biol.* **102**:15.

Sauerbier, W., 1964, Host cell reactivation of φX174 at intracellular UV irradiation, *Z. Vererbungsl.* **95**:145.

Schnitzlein, C. F., Albrecht, I., and Drake, J. W., 1974, Is bacteriophage T4 DNA polymerase involved in the repair of ultraviolet damage? *Virology* **59**:580.

Schopf, J. W., 1978, The evolution of the earliest cells, *Sci. Am.* **Sept**:111.

Seeberg, E., and Johansen, I., 1973, Incisions in ultraviolet irradiated circular bacteriophage λ DNA molecules in excision proficient and deficient lysogens of *E. coli*, *Mol. Gen. Genet.* **123**:173.

Sekiguchi, M., Yasuda, S., Okubo, S., Nakayama, H., Shimada, K., and Takagi, Y., 1970, Mechanism of repair of DNA in bacteriophage. I. Excision of pyrimidine dimers from ultraviolet-irradiated DNA by an extract of T4-infected cells, *J. Mol. Biol.* **47**:231.

Sekiguchi, M., Shimizu, K., Satao, S., Yasuda, S., and Ohshima, S., 1974, Enzymic mechanism of excision-repair in T4 infected cells, in: *Molecular Mechanisms for the Repair of DNA* (R. B. Setlow and P. C. Hanawalt, eds.). pp. 135–142, Plenum Press, New York.

Sertic, V., and Boulgakov, N., 1935, Classification et identification des typhi-phages, *C. R. Soc. Biol.* **119**:1270.

Setlow, J. K., 1962, Evidence for a particular base alteration in two T4 bacteriophage mutants, *Nature* (*London*) **194**:664.

Setlow, R., and Boyce, R., 1960, The ultraviolet light inactivation of φX 174 bacteriophage at different wavelengths and pHs, *Biophys. J.* **1**:29.

Setlow, R. B., and Carrier, W. L., 1964, The disappearance of thymine dimers from DNA: An error-correcting mechanism, *Proc. Natl. Acad. Sci. USA* **51**:226.

Setlow, R. B., and Carrier, W. L., 1966, Pyrimidine dimers in ultraviolet-irradiated DNAs, *J. Mol. Biol.* **17**:237.

Setlow, R. B., and Carrier, W. L., 1968, The excision of pyrimidine dimers *in vivo* and *in vitro*, in: *Replication and Recombination of Genetic Material* (W. J. Peacock and R. D. Brock, eds.), pp. 134–141, Australian Academy of Science, Canberra.

Shames, R. B., Lorkiewicz, Z. K., and Kozinski, A. W., 1973, Injection of ultraviolet-damage specific enzyme by T4 bacteriophage, *J. Virol.* **12**:1.

Siegel, E. C., 1973*a*, Ultraviolet-sensitive mutator strain of *Escherichia coli* K-12, *J. Bacteriol.* **113**:145.

Siegel, E. C., 1973*b*, Ultraviolet-sensitive mutator *mutU*4 of *Escherichia coli* inviable with *polA*, *J. Bacteriol.* **113**:161.

Simon, E. H., 1961, DNA synthesis in the development of resistance to ultraviolet irradiation in T2-infected *E. coli*, *Virology* **15**:237.

Simon, T. J., Smith, C. A., and Friedberg, E. C., 1975, Action of bacteriophage T4 ultraviolet endonuclease on duplex DNA containing one ultraviolet-irradiated strand, *J. Biol. Chem.* **250**:8748.

Sinsheimer, R. L., 1956, The glucose content of the deoxyribonucleic acids of certain bacteriophages, *Proc. Natl. Acad. Sci. USA* **42**:502.

Sinsheimer, R. L., 1959, A single-stranded DNA from bacteriophage ϕX174, *J. Mol. Biol.* **1**:43.

Sinsheimer, R. L., Starman, B., Nagler, C., and Guthrie, S., 1962, The process of infection with bacteriophage ϕX174. I. Evidence for a "replicative form," *J. Mol. Biol.* **4**:142.

Smith, E. V. P., and Jacobs, K. C., 1973, *Introductory Astronomy and Astrophysics*, W. B. Saunders, Philadelphia.

Speyer, J. F., and Rosenberg, D., 1968, The function of T4 DNA polymerase, *Cold Spring Harbor Symp. Quant. Biol.* **33**:345.

Stahl, F. W., 1959, Radiobiology of bacteriophage, in: *The Viruses*, Vol. 2 (F. M. Burnet and W. M. Stanley, eds.) pp. 353–385, Academic Press, New York.

Stair, R., 1969, Measurement of natural ultraviolet radiation: Historical and general introduction, in: *The Biological Effects of Ultraviolet Radiation* (F. Urbach, ed.), pp. 377–390, Pergamon Press, London.

Streisinger, G., 1956, The genetic control of ultraviolet sensitivity levels in bacteriophages T2 and T4, *Virology* **2**:1.

Sugino, A., Peebles, C. L., Kreuzer, K. N., and Cozzarelli, N., 1977, Mechanism of action of nalidixic acid: Purification of *Escherichia coli nalA* gene product and its relationship to DNA gyrase and a novel nicking-closing enzyme, *Proc. Natl. Acad. Sci. USA* **74**:4767.

Sussman, R., and Ben Zeev, H., 1975, Proposed mechanism of bacteriophage lambda induction: Acquisition of binding site for lambda repressor by DNA of the host, *Proc. natl. Acad. Sci. USA* **72**:1973.

Suzuki, K., Sato, E., and Morimyo, M., 1969, A mutant of *Escherichia coli* K12 exhibiting varying ultraviolet sensitivities depending on the temperature after irradiation, *Photochem. Photobiol.* **9**:259.

Symonds, N., Heindl, H., and White, P., 1973, Radiation sensitive mutants of phage T4, A comparative study, *Mol. Gen. Genet.* **120**:253.

Takagi, Y., Sekiguchi, M., Okubo, S., Nakayama, H., Shimada, K., Yasuda, S., Nishimoto, T., and Yoshihara, H., 1968, Nucleases specific for ultraviolet light-

irradiated DNA and their possible role in dark repair, *Cold Srping Harbor Symp. Quant. Biol.* **33**:219.

Takahashi, S., 1977, Joint molecules of λ DNA as an intermediate of genetic recombination, *Mol. Gen. Genet.* **150**:43.

Taketo, A., Yasuda, S., and Sekiguchi, M., 1972, Initial step of excision repair in *Escherichia coli:* Replacement of defective function of *uvr* mutants by T4 endonuclease V, *J. Mol. Biol.* **70**:1.

Tessman, E. S., 1966, Mutants of bacteriophage S13 blocked in infectious DNA synthesis, *J. Mol. Biol.* **17**:218.

Tessman, E. S., and Ozaki, T., 1960, The interaction of phage S13 with ultraviolet irradiated host cells and properties of the ultraviolet irradiated phage, *Virology* **12**:431.

Thakur, A. R., and Poddar, A. K., 1977, Growth and reactivation of single-stranded DNA phage φX174 *E. coli* undergoing thymineless death, *Mol. Gen. Genet.* **151**:313.

Tokunaga, F., 1975, Inhibitory effect of UV irradiation on the formation of twisted circular lambda DNA, *Int. J. Radiat. Biol.* **27**:237.

Tomilin, N. V., and Mosevitskaya, T. V., 1973, Reactivation and induction of c-mutation in UV-irradiated infectious lambda DNA resulting from UV irradiation of *rec+* host cells, *Mutat. Res.* **20**:429.

Tomilin, N. V., and Mosevitskaya, T. V., 1975, Ultraviolet reactivation and ultraviolet mutagenesis of infectious lambda DNA: Strong inhibition by treatment of DNA *in vitro* with UV-endonuclease from *Micrococcus lutens*, *Mutat. Res.* **27**:147.

Tomizawa, J., 1958, Sensitivity of phage precursor nucleic acid, synthesized in the presence of chloramphenicol, to ultraviolet irradiation, *Virology* **6**:55.

Tomizawa, J., and Ogawa, T., 1967, Effect of UV irradiation on bacteriophage λ immunity, *J. Mol. Biol.* **23**:247.

Trosko, J., 1970, Sunlight-induced pyrimidine dimers in human cells *in vitro*, *Nature (London)* **228**:358.

Unrau, P., Wheatcroft, R., Cox, B., and Olive, T., 1973, The formation of pyrimidine dimers in the DNA of fungi and bacteria, *Biochim. Biophys. Acta* **312**:626.

Van den Ende, P., and Symonds, N., 1972, The isolation and characterization of a T4 mutant particularly defective in recombination, *Mol. Gen. Genet.* **116**:239.

van de Putte, P., van Sluis, C. A., van Dillewijn, J., and Rorsch, A., 1965, The location of genes controlling radiation sensitivity in *Escherichia coli*, *Mutat. Res.* **2**:97.

van de Putte, P., Zwenk, H., and Rorsch, A., 1966, Properties of four mutants of *Escherichia coli* defective in genertic recombination, *Mutat. Res.* **3**:381.

Van Minderhout, L., and Grimbergen, J., 1975, A new type of UV-sensitive mutant of phage T4D, *Mutat. Res.* **29**:349.

Van Minderhout, L., Grimbergen, J., and DeGroot, B., 1974, Nonsense mutants in the bacteriophage T4D *v* gene, *Mutat. Res.* **29**:333.

Varghese, A. J., and Day, R. S., III, 1970, Excision of cytosine-thymine adduct from the DNA of ultraviolet-irradiated *Micrococcus radiodurans*, *Photochem. Photobiol.* **11**:511.

Varghese, A. J., and Wang, S. Y., 1967, Ultraviolet irradiation of DNA *in vitro* and *in vivo* produces a third thymine-derived photoproduct, *Science* **156**:955.

Wackernagel, W., 1974, UV-sensitivity and UV-mutability of infectious λ DNA: Reactivation, protection and mutability in various assay systems, *Mol. Gen. Genet.* **135**:61.

Wackernagel, W., and Winkler, W., 1971, A mutation in *Escherichia coli* enhancing the

UV-mutability of phage λ but not of its infectious DNA in a spheroplast assay, *Mol. Gen. Genet.* **114**:68.

Wallace, S. S., and Melamede, R. J., 1972, Host- and phage-mediated repair of radiation damage in bacteriophage T4, *J. Virol.* **10**:1159.

Weigle, J. J., 1953, Induction of mutations in a bacterial virus, *Proc. Natl. Acad. Sci. USA* **39**:628.

Weigle, J. J., and Delbrück, M., 1951, Mutual exclusion between an infecting phage and a carried phage, *J. Bacteriol.* **62**:301.

Wiberg, J. S., 1966, Mutants of bacteriophage T4 unable to cause breakdown of host DNA, *Proc. Natl. Acad. Sci. USA* **55**:614.

Wiberg, J. S., 1967, Amber mutants of bacteriophage T4 defective in deoxycytidine diphosphatase and deoxycytidine triphosphatase, *J. Biol. Chem.* **242**:5824.

Wilberg, J. S., Dirksen, M.-L., Epstein, R. H., Luria, S. E., and Buchanan, J. M., 1962, Early enzyme synthesis and its control in *E. coli* infected with some amber mutants of bacteriophage T4, *Proc. Natl. Acad. Sci. USA* **48**:293.

Willetts, N. S., and Mount, D. W., 1969, Analysis of recombination-deficient mutants of *Escherichia coli* K-12 carrying *rec* mutations cotransducible with thyA, *J. Bacteriol.* **100**:923.

Willetts, N., Clark, A. J., and Low, B., 1969, Genetic location of certain mutations conferring recombination deficiency in *Escherichia coli*, *J. Bacteriol.* **97**:244.

Winkler, U., Johns, H. E., and Kellenberger, E., 1962, Comparative study of some properties of bacteriphage T4D irradiated with monochromatic ultraviolet light, *Virology* **18**:343.

Witkin, E. M., 1947, Genetics of resistance to radiation in *Escherichia coli*, *Genetics* **32**:221.

Witkin, E. M., 1967, Mutation-proof and mutation-prone modes of survival in derivatives of *Escherichia coli* B differing in sensitivity to ultraviolet light, *Brookhaven Symp. Biol.* **20**:17.

Witkin, E. M., 1969*a*, The mutability towards ultraviolet light of recombination-deficient strains of *Escherichia coli*, *Mutat. Res.* **8**:9.

Witkin, E. M., 1969*b*, Ultraviolet-induced mutations and DNA repair, *Annu. Rev. Genet.* **3**:525.

Witkin, E. M., 1976, Ultraviolet mutagenesis and inducible DNA repair in *Escherichia coli*, *Bacteriol. Rev.* **40**:869.

Witkin, E. M., and George, D. L., 1973, Ultraviolet mutagenesis in *polA* and *uvrA polA* derivatives of *Escherichia coli* B/R: Evidence for an inducible error-prone repair system, *Genetics Suppl.* **73**:91.

Witkin, E. M., and Wermundsen, I. E., 1977, Induction of lambda prophage and of mutations to streptomycin resistance in separate small fractions of a lysogenic derivative of *Escherichia coli* B/r by very low doses of ultraviolet light, *Mol. Gen. Genet.* **156**:35.

Wolf, B., and Meselson, M., 1963, Repression of the replication of superinfecting bacteriophage DNA in immune cells, *J. Mol. Biol.* **7**:636.

Womack, F. C., 1965, Cross-reactivation differences in bacteriophages T4, *Virology* **26**:758.

Wood, W. B., and Revel, H. R., 1976, The genome of bacteriophage T4, *Bacteriol. Rev.* **40**:847.

Wu, J.-R., and Yeh, Y.-C., 1973, Requirement of a functional gene 32 product of bacteriophage T4 in UV repair, *J. Virol.* **12**:758.

Wu, J.-R., and Yeh, Y.-C., 1978*a*, New late gene, *dar*, involved in the replication of bacteriophage T4 DNA. II. Overproduction of DNA binding protein (gene 32 protein) and further characterization, *J. Virol.* **27**:90.

Wu, J.-R., and Yeh, Y.-C., 1978*b*, New late gene, *dar*, involved in the replication of bacteriophage T4 DNA. III. DNA replicative intermediates of T4 *dar* and a gene 59 mutant suppressed by *dar*, *J. Virol.* **27**:103.

Wu, R., and Yeh, Y.-C., 1975, New late gene, *dar*, involved in DNA replication of bacteriophage T4. Isolation, characterization, and genetic location, *J. Virol.* **15**:1096.

Wu, R., Ma, F.-J., and Yeh, Y.-C., 1972, Suppression of DNA-arrested synthesis in mutants defective in gene 59 of bacteriophage T4, *Virology* **47**:147.

Wu, R., Wu, J.-L., and Yeh, Y.-C., 1975, Role of gene 59 of bacteriophage T4 in repair of UV-irradiated and alkylated DNA *in vivo*, *J. Virol.* **16**:5.

Wyatt, G. R., and Cohen, S. S., 1963, The bases of the nucleic acids of some bacterial and animal viruses: The occurrence of 5-hydroxymethylcytosine, *Biochem. J.* **55**:774.

Yarosh, D. B., 1978, UV-induced mutation in bacteriophage T4, *J. Virol.* **26**:265.

Yarus, M., and Sinsheimer, R. L., 1964, The UV resistance of double-stranded ϕX174 DNA, *J. Mol. Biol.* **8**:614.

Yarus, M., and Sinsheimer, R. L., 1967, Ultraviolet sensitivity of the biological activity of ϕX174 virus, single-stranded DNA, and RF-DNA, *Biophys. J.* **7**:267.

Yasuda, S., and Sekiguchi, M., 1970*a*, Mechanism of repair of DNA in bacteriophage. II. Inability of ultraviolet-sensitive strains of bacteriophage in inducing an enzyme activity to excise pyrimidine dimers, *J. Mol. Biol.* **47**:243.

Yasuda, S., and Sekiguchi, M., 1970*b*, T4 endonuclease involved in repair of DNA, *Proc. Natl. Acad. Sci. USA* **67**:1839.

Yasuda, S., and Sekiguchi, M., 1976, Further purification and characterization of T4 endonuclease V, *Biochim. Biophys. Acta* **442**:197.

Yegian, C. D., Mueller, M., Selzer, G., Russo, G., Russo, V., and Stahl, F. W., 1971, Properties of the DNA-delay mutants of bacteriophage T4, *Virology* **46**:900.

Index