THE PHYSICS OF VIRUSES

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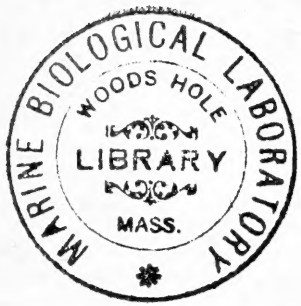

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FOREWORD

A child said *What is the grass?* fetching it to me with full hands;
How could I answer the child?

Interest in the physics of living systems, which was so active in the mid-eighteenth century when Mayer, Helmholtz, and Tyndall were leaders in contemporary thought, has passed through a relatively low period. The twofold reason has been the remarkable biochemical advance and the tremendous revolution in physics which started at the turn of the century—just as viruses were being discovered. These two fertile fields for research have kept biologists busy applying chemical ideas, and physicists busy reformulating the laws of nature. In the meantime students of viruses, using predominantly bacteriological methods, have uncovered a whole new form of living systems—ultimate parasites perhaps—which perform to a miracle the act of self-reproduction. As these objects took form, as filterable, stable, manageable preparations developed, physical studies on them began to be made. And as these studies began, the revolution in physics also ran its course. By 1933, when Stanley produced paracrystals of tobacco mosaic virus, the final form of quantum mechanics had been produced by Heisenberg, Schrödinger, Born, and Dirac. The physical theories underlying these chemical processes were complete by 1935. It is not surprising, therefore, that physics began to be applied to viruses to an increasing extent from that time on.

Now the physicist who approaches virus study feels two emotions. The first is the excitement of a challenge: viruses are at least representative of one form of life, perhaps the simplest. So far, except to insist that thermodynamics applies to life, the physicist has refrained from describing it in his own terms, and now a system comes into his thought where perhaps such a description is possible. Even more, the physical description may

be correct—and may fail, just as the high tide of Classical Theory in 1906 failed to cover the domain of the atom. So the challenge is real and welcome after weary computations verifying quantum theory in more and more elaborate systems. The second emotion is frustration: viruses are complex, understood only in terms of biological action, and the habits of starting in simple systems are seemingly not helpful. So, after a start, many a physicist has recoiled.

This book is an attempt to present what is known about viruses from the viewpoint of a physicist. In so doing, a major aim has proved to be the description of viruses, their shape and structure. Rather surprisingly, definite shapes and structures are emerging, and it is with a heightened sense of excitement that the hoped-for simplicity and symmetry are beginning to be found. So the account given here should interest not only the physicist but that growing body of students who admit to being virologists as well. And more—there is no doubt that the processes of viruses are in some way the processes of biology, so that the fundamental actions of biology may well be clarified by thinking about how viruses perform their work. It is significant that the author, starting as a physicist, has, through the study of viruses, developed an understanding of, and sympathy with, biologists, and heightened his already high respect for their penetrating discoveries. So biology and physics do in reality meet in this subject, and the era of Helmholtz and Mayer is once more returning.

Physical knowledge of viruses is advancing fast: every month sees an important piece of structural knowledge come within our ken. The methods outlined here are proving worth while, and the picture of viruses with the simplicity and beauty of Nature, herself, is beginning to be unveiled before our eyes.

The author wishes to acknowedge the great stimulus from all the members of the biophysics group at Yale. Argument and discussion there made the book possible. Also the liberality of Yale University in permitting a full-fledged nuclear physicist to deviate so considerably is here acknowledged, particularly the personal support given by Professor W. W. Watson. Thanks

are due to Mrs. Betty Jane Starr for her great help with the figures, to Mr. Donald Caspar and Mr. John Preiss for advice and help with some difficult parts, and to Miss Betty Hutchinson for typing the manuscript.

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CHAPTER ONE

THE NATURE OF VIRUSES AND THEIR RELATION TO PHYSICS

Introduction

Viruses are small objects which can produce the most devastating changes in certain living organisms. A bacterial virus which invades a bacterium of over a thousand times its size can in thirteen minutes have totally changed the functioning of the bacterium, and instead have produced three hundred replicas of itself. An animal virus, like small pox or influenza, can modify the total metabolism of a whole large animal, changing its mean temperature drastically, and producing physiological reactions requiring major changes in very large numbers of functioning cells.

The viruses themselves vary in size from 4,000 Å diameter for *psittacosis* to 100 Å diameter for foot and mouth disease. This last virus probably does not contain more than ten separately recognizable molecules, yet its net results on large animals are no more mild than many much larger and more complex viruses.

It is primarily because viruses possess in a remarkable degree one essential feature of life—the ability to reproduce—that they are exciting to study. Although a very great share of this ability resides in the host organism, the fact remains that a relatively simple and, in general, inert object can precipitate the formation of hundreds of like objects in a matter of minutes. How does it achieve this and what essential physical characteristics enable it to do so? These are the challenging questions of virus research.

Overwhelmingly the greatest part of virus research has been nonphysical in character. It has been concerned with virus-host

relationship, symptomatology, virus classification, growth of viruses in different species to produce attenuated viruses, immunological properties of viruses, chemical contents, and genetics. A very valuable and impressive body of knowledge has resulted. Nevertheless, the contribution of physics has not been small and is rapidly growing. The size and shape, degree of hydration, absorption spectra, and thermal properties of viruses are all being studied with very interesting results. Physics is beginning to tell something about the viruses themselves. Furthermore, viruses are so small that they represent one of the desirable extremes of study. If, indeed, the processes of the cell are by the present known laws of nature, by electrical and quantum-mechanical forces, by the processes of the molecular theory, it ought to be possible to see the action of these in the processes of virus multiplication. A very valuable start in this direction has been made by Puck, Garen, and Cline, who have shown the role of electrostatic forces in virus attachment to bacteria.

Therefore, even if the past direction of virus research has been primarily biological and chemical, it looks as though physical experimentation and theoretical speculation on viruses is well worth while. The aim of this book is to collect together the knowledge which bears on the physics of viruses in the hope that it can serve as a starting point for still more penetrating studies.

Because it is hoped that physicists and physical chemists will read this book, and because a summary is often a means of clarification, a brief outline of virology, slanted to suit our needs, is here given.

Outline of Virology

The first and most important fact about viruses is their apparent intimate concern with some host. So far, all attempts to secure virus multiplication on any kind of medium other than an intact organism have failed. This failure does not mean that all future attempts will fail, but it does mean that, whereas a plant will grow if supplied with water, air, light, and a few

inorganic nutrients, a virus seemingly needs a more complex and accurately balanced form of medium. It seems likely that the *physical distribution* of the material of the host cell, proximity to membrane or cell wall, etc., may be of importance. This is, as yet, unproven but it seems likely.

Because of this concern with a host, viruses are classified by their host. Thus we have tobacco mosaic virus, or an *E. coli* bacteriophage, or rabbit papilloma virus. There is, as yet, no definite and accepted classification of viruses, partly because no very definite means of distinguishing similar viruses exists.

All viruses, therefore, operate by invasion of a cell. This is a process which in itself merits much study. Once inside the cell, the virus is instantly concerned with the whole metabolic (chemical and energy turnover) process of the cell, which apparently shifts its function to serve the purpose of the virus, starting a process which ultimately yields a highly multiplied number of viruses. There is evidence from bacterial viruses that after invasion the virus ceases to have its normal structure and becomes something much less visible, and only later, in the multiple stage, does it become apparent in its normal condition.

Virus Multiplication

The unique nature of the bacterial host has enabled detailed studies of virus multiplication to be made. These studies probably apply to other viruses, but it must be remembered that much of our ability to generalize about viruses comes from the fact that we have, so far, made detailed studies on only a few. The process which occurs in a bacterial virus is the following. The virus first attaches, probably at the outset by electrostatic forces, but subsequently by a chemical surface action. No measurable multiplication takes place for a period known as the *latent period*, which is 13 min for T-1 *E. coli* bacteriophage, 26 min for T-2 coliphage, and 60 min for M-5 *B. megaterium* phage. At the end of this time, lysis, or dissolution, of the bacterium occurs, and a number, varying from 50 to 1,000, of new virus particles are released. This process is called a *burst*, and the number the *burst size*. Both the latent period and burst

size are to some extent dependent on the previous history of the virus. A virus which has been dried and heat treated has a longer latent period and a smaller burst size.

Various agents can be used to estimate the number of virus particles present at any stage of this process. The larger phages can be seen as scattering centers in the ordinary microscope. The number of these drops from one, at the original moment of invasion, to zero for the first 95% of the latent period. In the last 5%, many scattering centers are seen, and these correspond to the number of virus particles released. Or the cellular processes can be stopped with proflavin, the cells forcibly burst open by pressure, and the contents examined with the electron microscope. The results of this kind of study show that, at about 85% of the latent period, incomplete virus particles are present—doughnut heads in place of barrel-shaped heads, for example. Thus, virus assembly would appear to be a relatively late and correspondingly rapid process. Similar conclusions can be reached by bombarding infected *E. coli* with X-rays at various stages of the latent period. Only toward the end of the latent period does a multiple-hit type of survival curve become apparent, again indicating that virus assembly is a late process. This process of assembly is not understood—the reader must see that it represents a tremendous intellectual challenge.

Size and Shape of Viruses

This will be the subject of detailed consideration later. The shortened list in the table on page 5 gives an idea of the range of sizes covered by viruses. The unit used is the Ångstrom, 10^{-8} cm, which is the experimental unit used in describing atomic and molecular sizes.

The objects we deal with, therefore, vary in diameter over a factor of 45, and in mass by a factor of roughly 10^5. One might, therefore, be on guard regarding treating viruses as all alike, and no attempt will be made to do so here.

Not nearly enough is known of the true shape of viruses. They seem to be constructed, very broadly speaking, out of spheres and rods. Only for four plant viruses—tobacco mosaic, southern

Virus	Diameter or appropriate size (Å)
Psittacosis	4,500
Vaccinia	2,100 × 2,600
Herpes simplex	1,500
Influenza	1,150
T-1 bacteriophage	500; tail 1,200
Southern bean mosaic	310
Bushy stunt	260
Lansing polio	250
Foot and mouth disease	100
Tobacco mosaic	2,800 × 120
Transforming factor r → III pneumococci	3,400 × 85

bean mosaic, tobacco necrosis, and tomato bushy stunt—are the shapes in solution or suspension accurately known. Of these, the first consists of long rods, 2,800 Å, or more, in length, with probably a hexagonal cross section 120 Å across. The other three are very accurately spherical. Electron micrographs of dried viruses abound, and, from these, three shapes seem to cover all observed classes: roughly spherical; rod-like; or sperm-like, with nearly (but not accurately) spherical heads and quite long tails. It is very unwise to suppose that we know enough to generalize as yet.

Mutation of Viruses

Viruses are apparently capable of definite mutation. Various means for recognizing these exist, for example, in terms of the symptoms they produce or in terms of the range of host in which they will multiply. There are supposedly 50 types of tobacco mosaic virus.

Virus mutations are found in various ways. The usual mutagenic agents are effective on viruses, notably ultraviolet light, chemical mutagens, and thermal action. The application of genetic methods of study to viruses is perhaps the most powerful presently available technique. A multiple infection of a bacterium with two different mutants of a bacterial virus yields differentiatable progeny from which many genetic facts can

be deduced. Thus T-2 phage has three linkage groups, and at least 15 distinguishable genes. This will be discussed in more detail later.

Mutual Interference of Viruses

Two wholly unrelated viruses, which operate in two different classes of cell, seem to be capable of separate multiplication, so that a joint infection of a large organism containing both types of cells will yield some of each in the end. However, if two viruses can grow in the same cell, they interfere with one another (unless they are very closely related) so that from a mixed strain only one will develop. However, in a multicellular organism there may be "victory" by one virus in one cell and the other in the other cells. Thus a mixed strain of two viruses may stay mixed after an infection, but the properties of the two are likely to be changed. Very closely related strains, such as two mutants of the same virus, will give a mixed yield. This applies to very similar viruses in some cases, such as T-2 and T-4 *E. coli* bacteriophage. T-1 and T-7, which are morphologically distinct, always mutually interfere.

Virus Attenuation after Multiple Passage

A very important feature of applied virus research is the production of attenuated strains which have lost their virulence for one host, and maintain the ability to multiply harmlessly. Such a virus can produce full immunological protection against a later invasion by a dangerous virus and is obviously a very desirable agent in disease control.

The procedure used is to try to find secondary hosts, notably chick embryos, which will survive enough to permit serial passages. This is kept up, and every so often the virus is tried in the original host. Very remarkable results are found. In some cases, after several hundred passages, the desired strain is produced. In other cases, although a virus which is not pathogenic in the new host can be developed, a full return to virulence after one passage in the old host is observed.

It is hard to be very definite as to what this process actually

is. According to the present all-pervading genetic doctrines, the attenuation is the selection of a particular virus strain from a mixture. Thus, any fresh virus preparation is thought of as perhaps containing thirty or so different strains. By serial passage through a well-chosen host, the proportion of these can be so altered that the virus is predominantly of a virulent strain. This way of explanation is not necessarily what one would choose *ab initio* from the facts, inasmuch as modification can still be observed after 300 serial passages.

Chemical Composition of Viruses

In order to determine this, quite pure preparations are necessary. This is relatively easy in the case of larger viruses, but very hard for smaller. So no great claim to precision should be made. A table, taken from Stanley and Lauffer (1948), of the elemental composition, and of the proportion of protein, lipid, and nucleic acid, is given below. It is nearly true that only animal viruses contain any lipid.

Virus	C	H	N	P	S	Protein	Nucleic acid	Lipid
Southern bean mosaic	45.6	6.5	17	1.9	1.3	79	21	—
TMV	51	7.6	16.6	0.6	0.2	94	6	—
Tomato bushy stunt	48	7.7	16.1	1.4	0.6	83	17	—
Rabbit papilloma	50	7.2	14.8	0.9	2.2	89.5	9	1.5
Influenza	53		10.0	0.9		67	5	23
Newcastle disease	51.8		9.9	0.9		67	6	27
Vaccinia	33.7		15.3	0.6		83	5.6	4
T-2 phage	42		13.5	5.0		50	45	2

In the case of tobacco mosaic virus (TMV) and cucumber virus, an analysis of the amino acid content of several strains has been made. This is given in the table on page 8. (See Knight 1947.)

It can be seen that the differences between the TMV strains are not marked.

It is remarkable that 90% of the surface amino groups on tobacco mosaic can be acetylated without affecting the infec-

Amino Acid Content of Tobacco Mosaic Virus

Amino acid	TMV	Masked	Green aucuba	Yellow aucuba	Cucumber virus 3	Cucumber virus 4
Alanine	5.1	5.2	5.1	5.1		6.1
Arginine	9.8	9.9	11.1	11.2	9.3	9.3
Aspartic acid	13.5	13.5	13.7	13.8		13.1
Cystine	0.69	0.67	0.60	0.60		0.0
Glutamic acid	11.3	11.5	11.5	11.3	6.4	6.5
Glycine	1.9	1.7	1.9	1.9	1.2	1.5
Isoleucine	6.6	6.6	5.7	5.7	5.4	4.6
Leucine	9.3	9.3	9.2	9.4	9.3	9.4
Lysine	1.47	1.95	1.45	1.47	2.55	2.43
Phenylamine	8.4	8.4	8.3	8.4	9.9	9.8
Proline	5.8	5.9	5.8	5.7		5.7
Serine	7.2	7.0	7.0	7.1	9.3	9.4

tivity. It seems as though something involving arrangement, rather than chemical content, is of great importance.

Virus Serology

Viruses are excellent antigens, and, accordingly, the serological behavior of viruses gives us an excellent method of study of viruses, particularly as regards their surface properties. To illustrate the potency of viruses as antigens, a preparation of infectious sap of a plant virus, which has in no sense been thoroughly purified, is antigenic primarily because of the virus and not because of the unremoved cell constituents also present.

It is unfortunate that we do not yet know how antibodies are produced in response to antigens. It is not a simple physical process, for antibodies can continue to be formed after the original antigen molecules have disappeared. Yet there seems to be an underlying simplicity about the end results, for the result of injecting antigen into an animal is to produce molecules in the blood serum which are clearly related to the original antigen molecules and have for them a strong and specific affinity. Both the affinity and its specificity can be used in virus study, and a very great branch of virus research proceeds along serological lines.

The technique is as follows. As pure a preparation as is practical is injected into the serum-producing animal (for example, a rabbit or horse) in three doses a few days apart. At the end of three weeks, the animal is bled. The serum is separated by spinning out the blood cells, and for crude purposes can be used as an antiserum. It is usual to purify the antiserum further by adding specific substances known to be present in the original preparation, for example, plant sap from healthy plants, or bacterial debris from broken-up bacteria in the case of bacterial viruses. These cause precipitation of the antibodies specific to them and leave a more specific antibody to the virus itself.

Two standard methods of measuring serological combining power, or affinity, are in general use. The precipitin method relies on the observation of a visible precipitate after the addition of antibody to virus; the neutralization of infectivity utilizes the fact that, after combination, the infectivity is removed or reduced.

Virus serology will be taken up in much more detail later. It can be said now that viruses have of the order of 1,000 antigenic units on their surface; that several different types of unit exist per virus; and that the units differ in size, ranging from the equivalent of eight or ten amino acid side chains, to whole protein molecules. The parts of a bacterial virus which cause attachment to a bacterium do not seem to be active antigens, although this can not be asserted too vigorously.

Hemagglutination

An interesting property of a fair number of animal viruses, such as influenza, Newcastle disease, vaccinia, and mumps, is that of causing red blood cells to clump together, or agglutinate. This is in some cases due to substances called agglutinins, which are capable of separation from the virus, and in other cases due to units in the virus itself. An interesting feature of some of these viruses is the property of self-elution, by which is meant that after a while the virus will remove itself from the red cell and agglutination will cease. After this process has occurred, the red cells will not longer agglutinate.

This is interpreted, at present, as follows. The red cells are supposed to have receptors on them which can attach to the virus. The self-eluting virus attaches at a point which is enzymatically active on the receptor as substrate. The enzyme then slowly converts the receptor into some noncombining form, which, therefore, ceases to be bound by the virus, and so the process of elution can take place. Such red cells may resuspend, but they can no longer be acted on by the same virus to agglutinate.

Purified Virus Preparations

In the case of the plant viruses, notably tomato bushy stunt, tobacco necrosis, and southern bean mosaic virus, purified preparations which can be crystallized into crystals of definite form have been prepared. The procedure is elaborate, although one difficulty is simply in starting from enough infected plants to give a high yield. Tobacco mosaic virus forms small, visible crystalline aggregates.

No crystalline preparations of animal or bacterial viruses have, at the time of writing, been prepared. The concentration of bacterial viruses can be raised to about 10^{13} per cm^3 by careful growing and fractional centrifugation. However, physical studies on bacterial virus are still somewhat limited by the lack of a pure preparation.

Virus Assay

Of predominant importance in virus assay is the use of dilution and some all-or-nothing effect, like producing the proper symptoms in a host, or an agglutination of red blood cells. Any given sample containing virus is diluted in an appropriate suspension medium (buffer or broth for example) by known amounts, e.g., in factors of 10, and each dilution is tested for the required effect. If a dilution of 10^6-fold, for example, will still produce symptoms, this number is often used to describe the *virus titer*. To a physicist this may seem to be a very crude measurement. Actually, for many purposes, only the logarithm of the virus titer has any significance, and when this is so, the accuracy is

not so bad. Lack of accuracy did not prevent the founders of modern physics from measuring the charge on the electron, or Avogadro's number, accurately enough to move forward fast in that subject. The physical study of viruses is today historically where atomic physics was in 1900.

This dilution technique is often all that is available for animal virus work. For bacterial viruses a very powerful method is available. The idea used exploits the fact that virus infection of a bacterium results in dissolution, or *lysis*, of the bacterium, producing a clear solution. If agar jelly, nutrient medium, and bacteria are mixed together and poured into a thin layer on a glass petri dish, the result will be a whitish, cloudy growth of bacteria. If a *small* number of virus particles are added to a similar mixture and it is poured, then, after about 8 hr, the cloudy growth will appear, but around each virus there will be a clear spot, or plaque, which is readily seen. The number of these plaques is, then, an exact count of the viruses. This is a method of tremendous sensitivity, comparing very favorably with any of the techniques of physics. As will be seen later, many elaborations of this means of assay can be devised. Of course, to cover a wide range, dilution must also be employed, but this can be done quite accurately.

For plant viruses, a method which is rather similar in character, but unfortunately not so simple or accurate, is available. This is the method of local lesion counting. Some host plants are so sensitive to virus infection that, as the virus spreads, the plant is locally killed, and the infection then ceases because it has been over-efficient. The killed patches, or local lesions, are then easily seen and counted. The difficulty in the method lies in plant variability and in the serious problem of cell invasion. Virus will not penetrate a plant cell until it is damaged, and the damage must not be too severe or the cell dies. The technique used is to rub a little fine Carborundum powder over the leaf and then follow with a smearing of the virus solution to be assayed. Half leaves can be used for comparison. In addition, plant viruses show a marked tendency to aggregation. The theoretical basis for lesion counting is discussed by Bald (in

Viruses 1950), and he concludes that y, the number of lesions, is given by

$$y = N(1 - e^{-pvx})$$

where N is the number of points at which infection could occur, pv is the average number of virus aggregates which enter the possible infection points, and x is the virus concentration. Thus, in practice the procedure involves plotting an experimental curve of y versus x on the same test plants, and using this to determine an unknown, x.

It is always assumed, and probably justifiably so, that a single virus can cause infection.

Lesion counting is possible for animal viruses, the chorio-allantoic membrane of a chick embryo being suitable for use.

Where any virus is characteristically recognizable in the electron microscope, and where high enough concentrations are available, this direct method of assay can be used.

Origin of Viruses

Here we are in deep waters. In the first place, it is not yet the part of the physicist to describe the origin of a living thing, or even something related to it. In the second place, there is no general knowledge bearing on the origin of viruses. In the one case of bacterial viruses, which may not be typical at all, the phenomenon of *lysogenesis*, discovered by Listome and Carrere, Bordet, and Burnet, seems to push one stage back toward the origin of viruses. Certain bacteria, notably *B. megaïerium*, carry a pro-virus, which is manufactured at each cell division and carried by each bacterium. Under the influence of ultraviolet light, or of certain reducing agents, the pro-virus develops into active virus, which then causes bacterial lysis, whence the name *lysogenic*. The pro-virus is presumably an active, useful part of the bacterial cell, which carries the capability of change into a dominant destructive object. Studies of the pro-virus are progressing rapidly, and it is one of the most exciting branches of virology at the moment.

The implication of the pro-virus is that all viruses originate in host cells, either of the final host or one like it. We can, there-

fore, claim that viruses probably represent, in a distorted way, the operation of certain cell components. This makes the study of viruses of interest because it may be attacking, in simple form, the problem of single-cell constituents.

Physics and the Study of Viruses

The kind of physical methods which may pay off in virus studies are hardly likely to be those of classical physics, involving electrical properties, stress, strain, specific heat, and so on. Rather, the methods of modern physics need to be used. A moment's thought shows that modern physics has progressed by three broad techniques—bombardment, and observation of the consequences, as used in nuclear physics and in atomic-energy-state work; absorption and emission of radiation, as in spectroscopy of all wavelengths; and scattering of radiation, either as scattering or as diffraction. It is worth a moment to see how these broad techniques can be applied to virus study.

The methods of nuclear physics—bombardment, and observation of changes—are clearly applicable. Indeed the very same particles—protons, deuterons, alpha particles, and electrons—prove to be highly suitable. The changes produced are not transmutations, but are loss of infectivity, of serological affinity, of absorptive ability, killing power, and interfering power. To each of these a measured *cross section* can be assigned, and, as in nuclear physics, the cross section must then be interpreted theoretically. This kind of work is just beginning.

The second technique—absorption and emission of radiation—is obviously confined to absorption. This requires pure virus, and, as it has become available, valuable information regarding ultraviolet absorption has been obtained. In some cases this can be combined with polarization studies. The refinement of absorption work includes low-temperature absorption, and very interesting results can be obtained in this way. The great sensitivity of virus assay enables a second feature of absorption of radiation—loss or change in function—to be measured. This type of study—called the plotting of an action spectrum—is also only in its infancy.

The third technique—scattering and diffraction—again needs

pure preparations, and has been exploited to some extent. The rewards are size and shape figures and, occasionally, internal structures.

In addition to these, the two features possessed by viruses—large size on the molecular scale, and sensitivity of biological properties—enable sedimentation studies in centrifugal fields and diffusion studies to be made, and thermal and pressure effects to be measured.

Now, none of these *alone* can be taken as finally informative, because the essential character of work in the unseen submicroscopic world is that it is *inferential*. Therefore, as in modern physics, we must wait for agreement between several lines of work before stating firm conclusions. This is already beginning to happen. For example, the size and hydration of southern bean mosaic virus, determined by sedimentation and diffusion, agrees quite well with measurements made in the electron microscope and by small-angle X-ray scattering. The electron microscope data are further checked by deuteron bombardment studies. So the interplay of varied information, which has built atomic physics wholly in the unseen world, is beginning to take effect in the physical studies of viruses. The function of this book is to show where these studies are leading us, and to what extent the physical method has power.

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CHAPTER TWO

THE SIZE, SHAPE, AND HYDRATION OF VIRUSES

Very great advances in our knowledge of the size and shape of viruses have been made in the past 15 years. Viruses are no longer characterized as vague, filterable pathogenic agents but today are thought of as having a definite physical shape, which in one or two cases is very accurately known. A large part of this advance has been due to the ability to prepare virus that is sufficiently concentrated to be seen with the electron microscope, but other methods have also played a part. In this chapter, we discuss the various available methods and give the more striking results. A summary of methods of measuring virus size was given by Markham, Smith, and Lea (1942).

Optical Microscopy

Ultraviolet microscopy, using either reflecting or quartz optics, can be carried out down to a wavelength of 2,000 Å. This means that resolution to about 2,000 Å is possible, and some viruses are sufficiently large to be seen in this way. Psittacosis can be seen readily and its size determined; vaccinia can just barely be seen, not adequately for confirmation of its shape. In actual fact, modern techniques have not been applied sufficiently to this old problem. The great advantage of optical microscopy lies in the fact that the virus does not have to be dried and put in a vacuum, and hence an idea of its shape under living conditions can be obtained.

Electron Microscopy

The electron microscope has been of great value in virus research. No matter how determined a virologist may be to

leave electron microscopy to other experts, the day soon comes when he is making concentrated preparations and waiting for the visual knowledge which the electron microscope can give. There is no effective limit to the resolution of the electron microscope, and without doubt this instrument dominates the whole field of study of virus size. As ordinarily used, 50-kv electrons are employed. These are focused on the virus preparation, which is deposited on a thin collodion membrane held on a fine wire mesh. It is usual to shadow the preparation with chromium, gold, or uranium to provide areas which are definitely opaque to the passage of electrons. Such a shadowing technique has the advantage of providing a third dimension which is dependent on the thickness of the virus and on the angle of shadowing.

Shadowing technique consists of placing the collodion film preparation in a high vacuum (10^{-5} mm mercury, requiring an oil or mercury diffusion pump) together with a heater containing the element used for shadowing. This may consist of a tungsten metal strip with an indent to take the evaporated metal, or may simply be a coil of tungsten wire with chips of metal held loosely in the coil. After the vacuum is established, the tungsten is heated by passing a current through it, and the metal atoms then evaporate. If the vacuum is excellent, the atoms make no collision on the way to the virus preparation and accordingly produce an accurate geometrical shadow just as would be seen for oblique light. The spaces in the shadow are transparent, and the focusing action of the microscope produces an image which gives an idea of the shape and thickness of the virus. By using comparison latex particles of known size, the microscope can give fairly accurate dimensions. The method has been applied to many viruses, notably the T-series bacteriophages, tobacco mosaic virus, southern bean mosaic virus, vaccinia, influenza, and polio viruses. In Fig. 2.1 are shown electron micrographs of T-1 bacteriophage, southern bean mosaic virus, and tobacco mosaic virus, taken by Dr. D. J. Fluke.

In view of the success of this technique, some of its shortcomings need to be discussed. The greatest of these lies in the

fact that the virus has to be studied in a high vacuum where it loses a great many of its structural features as it loses its unbound water of hydration. Thus it is quite possible that many characteristic features of viruses as seen in electron micrographs are actually produced in the drying process and are not real.

Also, it has been pointed out by Anderson (1950) that in drying a preparation the virus is exposed to strong surface forces. He has overcome this by the ingenious method of suspending the object to be viewed in liquid CO_2 or N_2O (which unfortunately requires very high pressure) and then raising the temperature past the critical point, thus avoiding exposing the virus to passage through a meniscus. The N_2O is then allowed to escape, and the Formvar membrane is recovered from the bottle and used for microscopic observation. Before this can be done, water must be replaced by a series of other solvents. Viewed in this way, preparations do seem different, notably they are less flattened down.

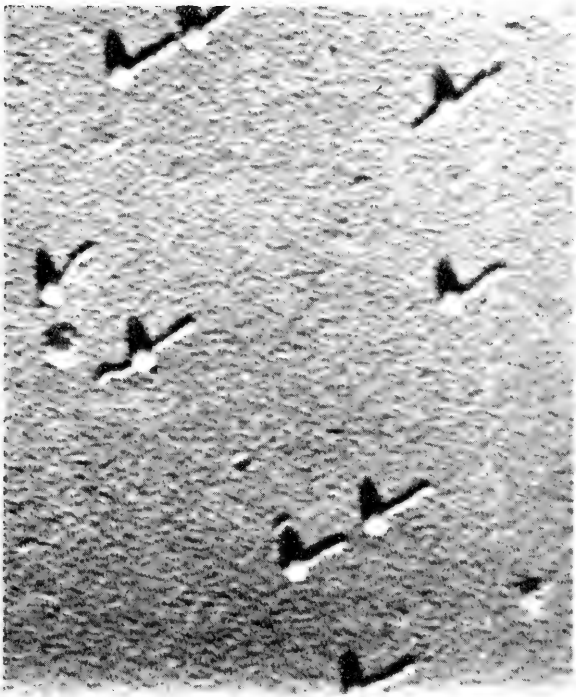
Another disadvantage of high-magnification microscopy is the high concentration needed to guarantee that even one particle will appear in the field. About 10^{11} particles cm^3 are necessary, so that one useful adjunct of virus microscopy is a high-speed centrifuge for concentration. This process, in turn, may alter the characteristics of the virus.

Many viruses, notably vaccinia and some phages, show definite signs of internal structure. These represent a first step in establishing the internal structure of viruses, a subject which is just starting. Correlation between these structural units and virus function is most desirable but has not yet been successful.

Viruses of very characteristic shape, like tobacco mosaic virus, can readily be counted with the electron microscope. This secondary use is very valuable in establishing growth curves for plant viruses.

Electron microscopy, in the hands of experts, can give considerable information about details of structure. For example, Williams (1952) has subjected tobacco mosaic virus to sonic disturbance, thus breaking the virus rods into shorter sections which can occasionally stand on end. When these are shadowed

(a)



(b)

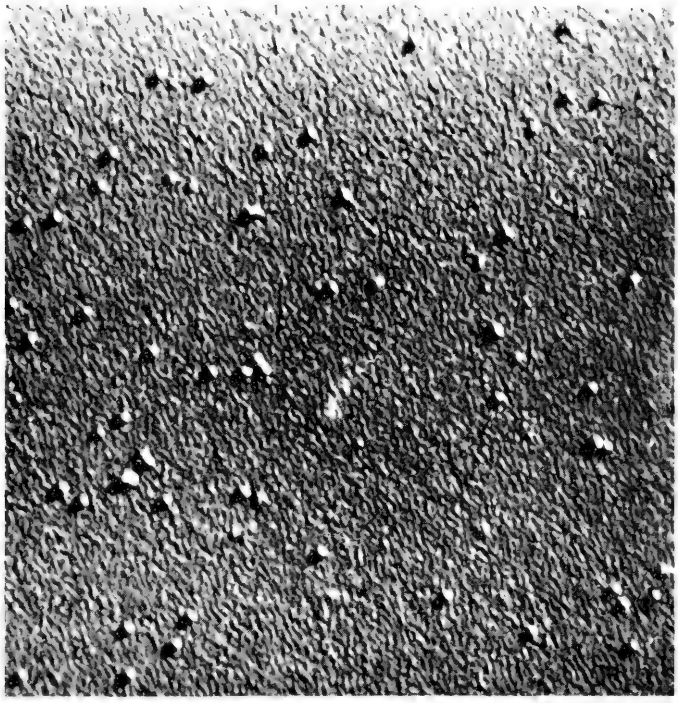


FIG. 2.1, see p. 19.

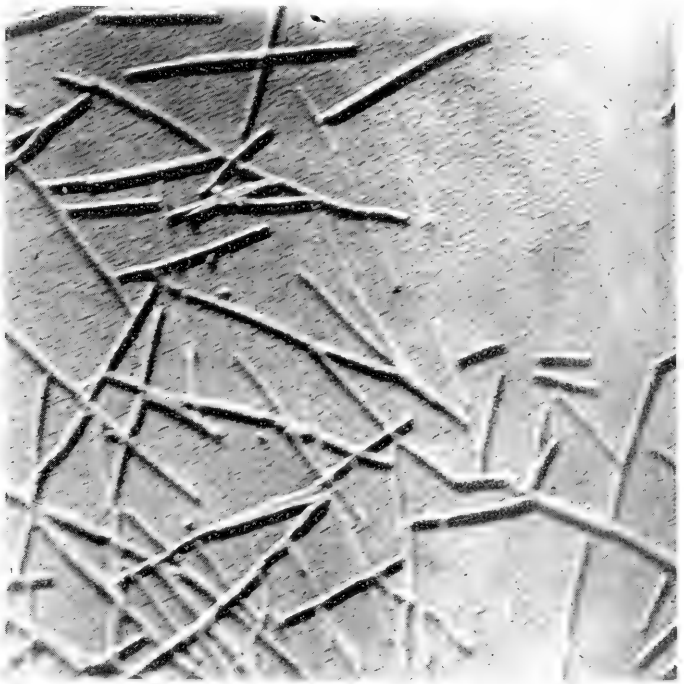


FIG. 2.1. Electron micrographs of viruses: (a) is T-1 bacteriophage, (b) is southern bean mosaic virus, and (c) is tobacco mosaic virus. These were taken by Dr. Fluke at Brookhaven National Laboratory.

and observed, the nature of the cross section of the virus is seen to be hexagonal with a side of length 87 Å. One such electron micrograph is shown in Fig. 2.2. There is a considerable future for this combination of physical disturbance and subsequent electron microscopy.

One feature of electron microscopy which is of great importance is the observation of the virus in association with the host organism. This is possible for bacterial viruses and also, by the technique of embedding a tissue section in methacrylate and then cutting thin sections, can be applied to plants and animal viruses. The section technique was described by Neuman, Borysko, and Swerdlow (1949), and if metal shadowing to prevent the development of charge on the slice is used, excellent micrographs are obtained (Mühlethaler, 1952). An example of

work of this kind is the study of tobacco mosaic virus within Turkish tobacco plants by Black, Morgan, and Wyckoff (1950). Tobacco mosaic virus is very suitable for such studies as it has a characteristic long, thin particle shape and so can be recognized readily. It was found that in diseased cells the long, rod-

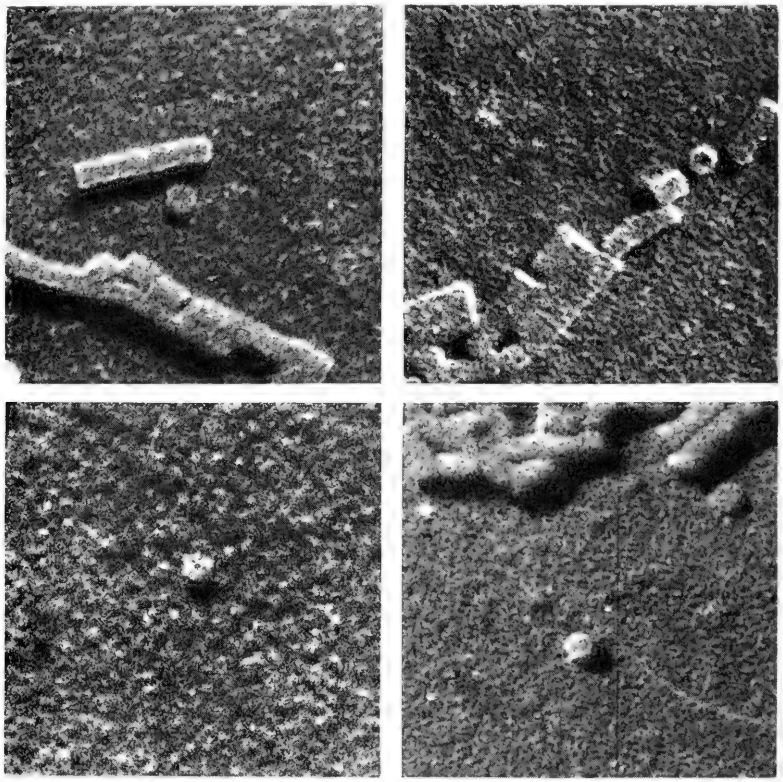


FIG. 2.2. Electron micrograph of sonically treated tobacco mosaic virus, taken by Dr. R. C. Williams, showing one fragment standing on end. The hexagonal cross section is visible. Magnification 200,000.

shaped particles grow inward from the cell wall and can completely fill the space between the wall and the nucleus. The nucleus does not appear to develop the characteristic virus rods. Also, the length of rod seen in the plant cytoplasm is not the short rod of 2,800 Å length usually associated with the infectious unit but is much longer. Since crystalline inclusion

bodies are observed in the hair cells of *Nicotiana tabacum*, and these are infective (Bawden, 1950, p. 44), these observations are a confirmation of the extensive internal cellular growth of a plant virus.

Very interesting information about the nature of influenza virus and of Newcastle disease virus has been obtained by this method. Wyckoff (1951) has examined sections of the chorioallantoic membrane of chick embryos infected with PR-8 influenza virus. He found filaments extending outward from the membrane with spherical particles apparently associated with, and perhaps being capable of development from, the filaments. Since the appearance of ultracentrifugally "purified" virus shows spherical particles (which are infectious), the question is raised as to whether the spherical particles are actually in spherical form during the process of multiplication. Similar results were found for Newcastle disease by Kilham, Morgan, and Wyckoff (1951). Filaments growing out into the chorioallantoic fluid were observed with some spherical particles possibly associated with them.

Very beautiful pictures of the curious mosaic of vaccinia in the same chick-embryo membrane have been obtained by Wyckoff (1951). Again, the striking feature of the pictures is the great extent to which the cytoplasm is manufactured into virus particles.

The effect of the combination between two spherical plant viruses and specific antiserum has been studied by Black, Price, and Wyckoff (1946). The virus particles can be seen to be in microflocculent aggregations with about twice their normal separation.

A very important series of electron-micrographic studies of plant viruses in unpurified extracts has been made by Bawden and Nixon (1951). In the following cases they found no specific particles: tomato spotted wilt, potato leaf roll, sugar beet mosaic, sugar beet yellows, cauliflower mosaic, and tomato aspermy virus. They could not have detected less than 10 mg/l if spheric shaped, or less than 1 mg/l if rod shaped. For potato virus X, they observed rod-like, tenuous particles 100 Å wide

and of greatly variable lengths. Similar results, but in lower concentration, were observed for potato virus Y, potato paracrinkle, henbane mosaic, tobacco etch, and cabbage blackring. For cucumber mosaic virus, they observed variable length rods 150 Å wide in about one-thousandth the concentration of tobacco mosaic virus. Spherical viruses were observed for tobacco ringspot (260 Å diameter) and Rothamsted tobacco necrosis, which showed two sizes of particle 180 Å and 380 Å in diameter.

The development of the growth of psittacosis virus is chorioallantoic membranes has been followed by Heinmets and Golub (1948). Definite development could be seen. The size at 72 hr was 3,600 Å diameter.

Valuable as is this kind of work, it suffers from the disadvantage that an enormous amount of understanding of cellular constituents is required before confident assertions can be made. After all, the electron microscope permits observation to nearly 100 times the fineness of detail of the optical microscope, and this means, then, thousands of times in area. All of this is largely unexplored and undescribed. Besides this complication, the preparative techniques of electron microscopy introduce different selections of special cellular constituents than optical microscopy, and, until all of these features are sorted out, the electron microscope must be used with care on tissue slices.

Ultrafiltration

This method has the outstanding advantage of universal applicability and does not need either highly purified preparations or specially high concentration. The method was started by Elford (1931) who used graded collodion membranes prepared by depositing collodion layers of different composition. In this way a graduation in pore size of filter can be obtained. To determine the pore diameter, a combined technique of viscous flow and fluid absorption is used. The filter is considered to be idealized as shown in Fig. 2.3. It is assumed that there are n cylindrical pores of radius r and length l. Then, in terms of Poiseuille's law of viscous flow, the volume, V, of liquid of

viscosity η flowing in a time t under a pressure difference P is given by

$$V = \frac{\pi}{8} nt \frac{1}{\eta} \frac{P}{l} r^4 \tag{2.1}$$

Everything can be measured as far as this expression is concerned except r^4 and n, so that we can consider a rate-of-flow

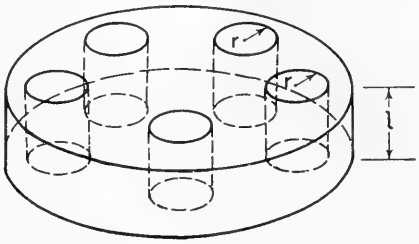


FIG. 2.3. Illustrating the principle of calibrating a collodion filter.

experiment as determining nr^4. By measuring the volume, v, of liquid absorbed and held by the filter, we have

$$v = n \pi r^2 l \tag{2.2}$$

The thickness, l, is then directly measured, and we have

$$V = \frac{vPr^2t}{8\eta l^2} \tag{2.3}$$

from which r can be found.

Markham, Smith, and Lea (1942) have subjected this to considerable examination. They conclude, from calibrations made with vaccinia, bushy stunt, hemocyanin, and hemoglobin, that the ratio of size of particle to average pore diameter is between 0.55 and 0.95, indicating, therefore, rather large limits of error. Stanley and Lauffer (1948) point out that as the particles and pore sizes both increase, the ratio approaches nearer to unity. Table 2.1 gives some comparative values. The units used are Ångstrom units.

A very good idea of the ultrafiltration method can be gained from the work of Melnick, Rhian, Warren, and Breeze (1951) who have applied it to Coxsackie virus and Lansing polio virus

TABLE 2.1

Virus	Filtration	Electron microscope	Sedimentation
Bushy stunt	130–200	260	275
TMV	150	150 × 2,800	150 × 2,800
Coxsackie Texas	150–230	350	330

and compared it with results from electron microscopy and sedimentation. The filtrate of "Texas 1" Coxsackie virus is markedly more infective when the average pore diameter exceeds 500 Å, and, from the actual point of onset of infectivity and the correction factor mentioned above, they conclude that the particle diameter is between 150 and 230 Å. The same virus was measured by sedimentation methods, as described in the next section, with the result that a value of 330 Å was obtained. An electron microscope figure for the same virus gave 350 Å. The values are included in the above table.

It can be seen that ultrafiltration can give valuable approximate values with initial preparations of new viruses. It would appear as though too much should not be expected of the method.

Observations of the Motion of Viruses: Diffusion, Sedimentation, and Viscosity

The use of the motion of an object in a force field and in a viscous medium formed the basis for early measurements of the electronic charge exemplified by Millikan's oil-drop experiment. The mobility of gaseous ions formed one part of the first discovery of structure in the atom. The same idea is used in studying macromolecules. The rate of diffusion, the viscosity, and the rate of drift under the action of a rotational acceleration are concerned with the size, shape, and hydration of a virus particle and so afford a method of studying these features.

Since all three of these observations of virus motion are important, and, if possible, all three should be made on any one virus preparation, a word about the interrelation of the three methods of study is first in order. In all three cases, a force is

applied to the virus: for the case of diffusion, the origin of this force is the osmotic pressure due to a difference in concentration; for sedimentation, it is the internal stress set up by rotational motion; and for viscosity, it is the force necessitated by a certain rate of shear.

Each of these has its own particular feature. Thus, the force in the case of diffusion does not depend on the density of the medium in which diffusion occurs, but it does depend on temperature. In the case of sedimentation in an ultracentrifuge, the applied force depends markedly on the density of the medium but is otherwise not temperature dependent. Viscosity is a matter of energy transfer through a medium and, therefore, depends essentially, on how much of the medium is occupied by the virus under study—this is not directly important in either of the previous cases. Viscosity studies are made with respect to the medium. For diffusion and sedimentation, the medium enters as the agent which provides a retarding force proportional to the velocity. This is in a different way from viscosity studies themselves.

Thus the three techniques, although really quite closely related, are actually capable of giving different and supplementary information about viruses. Rather oversimplifying, we can say that diffusion is concerned with size and shape, viscosity with number, size, and shape, and sedimentation with mass, size, and shape. We can now treat these three forms of study separately and later see how they can be combined.

Diffusion

The simplest way to visualize the diffusion of viruses is to think in terms of the osmotic pressure produced by viruses considered as large molecules sharing in the thermal agitation. If we consider a concentration, C, of virus per unit volume, the osmotic pressure exerted is $NkTC$, where N is the number of molecules per mole, k is Boltzmann's constant, and T is absolute temperature. Now, if C is varying with distance, x, the differential pressure over a distance dx is $NkTdC/dx$, and this is the excess force on a unit area of the space containing the

solute. If, as a result, the solute molecules move with a velocity v, then, by Stoke's law, the force due to viscous drag for a spherical particle of radius a is $6\pi\eta av$, where η is the coefficient of viscosity. For N_1 molecules per unit volume, we then have a total force on unit area of $6\pi\eta avN_1$, and this balances the force due to osmotic pressure. Thus

$$NkTdC/dx = 6\pi\eta avN_1 \tag{2.4}$$

Now, C is expressed in moles per unit volume, and if we substitute C_1 grams per unit volume we have $C = C_1/M_w$, where M_w is the mass of a mole of the solute. Then

$$\frac{NkT}{M_w}\frac{dC_1}{dx} = 6\pi\eta avN_1$$

But M_w/N is the mass, m, of one "molecule," and we then have

$$kTdC_1/dx = 6\pi\eta avN_1m$$

The mass, in grams, of N_1 molecules is N_1m, or M. The product of v and N_1m is the mass of solute flowing across unit area per second, or $vN_1m = dM/dt$. Then we find

$$\frac{dM}{dt} = \frac{kT}{6\pi\eta a}\frac{dC_1}{dx} \tag{2.5}$$

Now, the diffusion constant, D, is defined by Fick's law equation as

$$\frac{dM}{dt} = DA\frac{dC_1}{dx} \tag{2.6}$$

where A is area. Since we have considered unit area above, we derive from Eq. 2.5 that

$$D = \frac{kT}{6\pi\eta a} \tag{2.7}$$

This important result was derived by Sutherland in 1905 and applied by him to determine the molecular weight of egg albumin from Graham's diffusion data, he obtained the value 33,000.

If nonspherical viruses are concerned, then the frictional drag coefficient is no longer $6\pi\eta a$ but can generally be written as f, so that the more general equation for the diffusion constant is

$$D = kT/f \tag{2.8}$$

Diffusion constants, in practice, are measured by two methods. In the first, a sharp boundary is established by using a double cell, the upper part of which can be slid across until it forms an accurate continuation of the lower part. The two parts are filled separately, the lower with virus in buffer solution and the upper with buffer solution alone. When proper temperature equilibrium is established (the diffusion must be carried out under thermostatic conditions), the two parts of the cell are brought into line. The rate of diffusion can be measured by a refraction technique due to Lamm and Polson (1936) which consists of photographing a scale, by light from a mercury arc, through the cell. The boundary is marked by a change in refractive index, and this causes a shift in the apparent position of the scale. From the amount of shift at different positions in the cell, the distribution of virus can be estimated. Initially this is, of course, a step function, but, as diffusion progresses, the distribution becomes that of an error integral, and by evaluating the constant of this function as time passes, the diffusion constant can be obtained. Details are given by Neurath (1942). This method requires a rather elaborate, carefully made cell with a good optical system and a good thermostat. It also necessitates homogeneous, pure virus. Four determinations of diffusion constants have been made by this method: bushy stunt virus by Neurath and Cooper (1940), tobacco mosaic virus by Lauffer (1944), southern bean mosaic virus by Miller and Price (1946), and rabbit papilloma virus by Neurath, Cooper, Sharp, Taylor, Beard, and Beard (1941). Of these, there is excellent reason to believe that bushy stunt and southern bean mosaic viruses are accurately spherical, rabbit papilloma is probably spherical, and tobacco mosaic virus is definitely not spherical. The values obtained for the three spherical viruses, together with the derived radii, are given in Table 2.2.

TABLE 2.2
DIFFUSION CONSTANTS AND RADII FOR THREE SPHERICAL VIRUSES

Virus	Diffusion constant (cm^2/sec)	Radius (Å)
Bushy stunt	11.5×10^{-8}	187
Southern bean mosaic	13.4×10^{-8}	160
Rabbit papilloma	5.85×10^{-8}	368

Many classes of virus cannot be prepared in sufficiently high concentration for optical observation. This is notably true of bacteriophages. Polson (1944) has designed a simple and effective method for diffusion study which consists of a series of four superposed cylinders with holes drilled through them. The cylinders can be rotated so that the holes are all aligned, or they can be turned to separate the material in each cylinder. In this way the holes, which form the diffusion cell, can be filled, turned into place for diffusion, and then separated for sampling of the average concentration in the two layers. Polson has shown that if C_0 is the original concentration of the solution, C the mean concentration in the originally virus-free cell after time t, and H the height of the upper section, then D, the diffusion coefficient, is given by

$$D = \frac{\pi H^2}{t}\left(\frac{C}{C_0}\right)^2 \tag{2.9}$$

The measurement of the diffusion constant is thus a simple matter of comparing two concentrations. The ratio has to be accurately measured, as it occurs as its square.

Polson (1944) has applied this to equine encephalomyelitis virus, with the result that the diffusion constant is 8.0×10^{-8} cm^2/sec.

Polson and Shepard (1949) have studied T-3 and T-4 *coli* bacteriophages, with the result that at high concentrations T-3 has a diffusion constant of 1.19×10^{-7} cm^2/sec and T-4 a value of 8.0×10^{-8} cm^2/sec. T-3 is roughly spherical, with the possibility of a short tail. Assuming the spherical shape, the diameter deduced is 362 Å. No real estimate can be made for T-2,

which is not spherical at all. The same workers report an increase in diffusion rate at low concentrations. This is a surprising result, although not out of line with other protein diffusion data. In the case of protein diffusion, the increase is ascribed to dissociation, which seems to be rather difficult to apply to phage particles. More work needs to be done on this effect. Since the survival of a bacteriophage always requires the presence of a certain amount of synthetic medium, it would be worth while to repeat these experiments using specific serological affinity as the indicator.

Diffusion coefficients can also be measured through porous membranes. The difficulty of using these lies in the fact that the absolute diffusion coefficient is necessary, and therefore a preliminary calibration of the membrane is needed. This is not easy to do. In addition, charge effects, as described in Chapter 5, may introduce a complication.

Sedimentation

In the ultracentrifuge, the virus solution is placed in a cell which forms part of a rotor which may be driven in various ways. Leaving the technique aside for a moment we can consider what happens in the solution in the cell. The action of the motor and the suspension forces the whole rotor into circular motion. For this to occur, every point in the rotor has a varying inward acceleration $\omega^2 x$, where ω is the angular velocity in radians per second and x is the radius. In a solid, the force necessary to produce this acceleration is transmitted through the intermolecular forces as an internal stress which is adapted to provide the proper force. If adaptation cannot occur, the rotor bursts and a tremendous kinetic energy of the order of 5 ft-tons is released. Such explosions are very dangerous and, at the least, very frightening experiences.

When a liquid is in the cell the same force has to be applied, and it also takes place via the molecules of the liquid. However, if the liquid contains some molecules of greater density, the force necessary to hold these in place may be lacking, in which case the denser particles drift outwards. This outward drift

appears to be a centrifugal motion. The outward motion would be accelerated, but the acceleration is modified by the viscosity of the liquid, and so what occurs is a uniform rate of drift. This rate of drift is of the right size to provide the viscous force necessary to hold the particle in place, although slowly drifting. Thus, in the equation describing the motion we must take account of (*a*) the mass of the particle, (*b*) the density of the liquid, (*c*) the viscous drag, and (*d*) the angular velocity and radius.

Doing this, we get the following expression for the force acting on the particle, and which must be balanced by viscous drag

$$\text{Force} = x\omega^2 m - x\omega^2 V\rho$$

where m is the mass of the particle, V its volume, x the radius from the axis of rotation, and ρ the density of the liquid.

It is customary to express V in terms of the mass of the particle and the *partial specific volume*, V_0. This is defined as the volume increase produced in a large volume of solution by 1 gm of solute. When pure material is available, V_0 can be found by straightforward change in volume measurements. Using this idea, we have V, the particle volume, $= mV_0$. So the expression for the force to be balanced becomes

$$m\omega^2 x(1 - V_0\rho)$$

Now the viscous drag force is proportional to the velocity of drift and can be written as $f\,dx/dt$, so that the equilibrium drift will occur when

$$m\omega^2 x(1 - V_0\rho) = f\frac{dx}{dt} \tag{2.10}$$

This can be rewritten as

$$S = \frac{dx}{dt}\Big/\omega^2 x = \frac{m(1 - V_0\rho)}{f} \tag{2.11}$$

The quantity S is called the sedimentation constant, and if centimeters and seconds are the basic units, S is around 10^{-13} cm/sec per unit acceleration. The value 10^{-13} in such units is called a Svedberg unit and is designated by S.

The measurement of the sedimentation constant alone thus gives the ratio $m(1 - V_0\rho)/f$.

Particle Weight Measurement

The combination of diffusion and sedimentation offers a method of measuring the virus mass directly if the partial specific volume, V_0, is known. For we can combine the relations

$$D = kT/f \qquad \text{and} \qquad S = \frac{m(1 - V_0\rho)}{f}$$

to eliminate f and give

$$m = \frac{kTS}{D(1 - V_0\rho)} \tag{2.12}$$

In this expression, the buoyant term, $-V_0\rho$, corresponds to the reverse force due to the displaced water. Therefore, in principle, V_0 should be the partial specific volume of the dry virus, and m will then be the mass of the corresponding dry virus. The value of V_0 is accordingly found by measuring the increase in the volume, dv, of the virus solution due to the addition of a mass dm of virus. V_0 is then dv/dm. Using values obtained in this way, the results of Table 2.3 have been obtained.

TABLE 2.3
Masses of Viruses Determined by Sedimentation and Diffusion

Virus	Mass (gm)	Equivalent* molecular weight	Reference
Southern bean mosaic	1.09×10^{-17}	6,600,000	Miller and Price (1946)
Bushy stunt	1.7×10^{-17}	10,300,000	Lauffer and Stanley (1940); Neurath and Cooper (1940); McFarlane and Kekwick (1938)
Rabbit papilloma	7.0×10^{-16}	47,000,000	Neurath *et al.* (1941)
Tobacco mosaic	5.0×10^{-16}	30,000,000	Lauffer (1944)

* The equivalent molecular weight is the product of the particle mass and Avogadro's number, 6.03×10^{23}. This is more familiar to many, though it is doubtful if much is gained by thinking of a virus as a molecule.

In the case of spherical viruses, which are quite commonly found, the reasoning becomes relatively simple. For in this case, as for diffusion, the frictional drag is precisely described by Stoke's law and is $6\pi\eta a$, where a is the radius of the particle, and η the viscosity of the medium. We then have

$$S = \frac{m(1 - V_0\rho)}{6\pi\eta a} \tag{2.13}$$

Since m, the particle mass, is $\frac{4}{3}\pi a^3 V_0$, where V_0 is the partial specific volume of the particle, the problem of finding a reduces to finding the partial specific volume, V_0.

In this expression, the partial specific volume is that which applies to the unhydrated virus. This will not be valid if the density of the bound water is changed because of added forces on it, and so there is a little uncertainty here. However, the best procedure is to use the unhydrated value of V_0.

Doing this, we have

$$S = \frac{4}{3}\frac{\pi a^3(1 - V_0\rho)}{V_0 6\pi\eta a} = \frac{2a^2(1 - V_0\rho)}{9V_0\eta} \tag{2.14}$$

from which a can be determined.

The Frictional Drag Coefficient for Nonspherical or Hydrated Viruses

The simple case considered above is nearly always obscured by either asymmetry of the particles or by the presence of hydration. We can therefore look on the sedimentation process a little differently and solve the sedimentation relation for f, the frictional drag coefficient. Then

$$f = \frac{m(1 - V_0\rho)}{S} \tag{2.15}$$

and, if we write f_0 for the case where the particle is spherical, we have

$$f_0 = 6\pi\eta a = 6\pi\eta\left[\frac{3mV_0}{4\pi}\right]^{1/3}$$

The ratio f/f_0 is thus

$$\frac{f}{f_0} = \frac{m^{2/3}(1 - V_0\rho)}{6\pi\eta S\left[\frac{3V_0}{4\pi}\right]^{1/3}} \tag{2.16}$$

Replacing m by the value found from combined sedimentation and diffusion, we have, because $m = kTS/D(1 - V_0\rho)$,

$$\frac{f}{f_0} = \frac{1}{6\pi\eta}\left(\frac{kT}{D}\right)^{2/3}\left\{\frac{4\pi(1 - V_0\rho)}{3SV_0}\right\}^{1/3} \tag{2.17}$$

These relations are all set out in Svedberg and Pedersen (1940, Eqs. 68–70b). Actual values for the constants applicable to 20° C are given there.

It can be seen that, by employing Eq. 2.17, the results of sedimentation, diffusion, and partial-specific-volume measurement can, in the case of nonspherical viruses, yield a value of f/f_0, the ratio of the frictional drag coefficient to the equivalent-sphere frictional drag coefficient.

One obvious reason why f/f_0 should not be unity is the fact that the virus may have an asymmetrical shape. If this is known to be not the case—the virus is known to be spherical—one therefore predicts that the use of proper measurements together with Eq. 2.17 should give f/f_0 a value of unity. This is not found, and the explanation given in such a case is the presence of *hydration*, water which is bound on the surface and in the virus interstices so that the radius applicable to the frictional drag is not the radius of the dry particle. For the case of diffusion measurements, where the frictional drag term is all that is important, the effect of r gm of water (partial specific volume, V_1) combined with one of virus (partial specific volume, V_0) is to produce a frictional drag, f_D, where

$$\frac{f_D}{f_0} = \left\{\frac{rV_1 + V_0}{V_0}\right\}^{1/3} \tag{2.18}$$

In the case of sedimentation, the buoyancy term is also of importance, so the ratio to be applied as a correction factor for sedi-

mentation constants is f_S/f_0, where

$$\frac{f_S}{f_0} = \left\{\frac{(1 - V_0\rho)}{1 - V_0\rho + r(1 - V_1\rho)}\right\}\left\{\frac{f_D}{f_0}\right\} \tag{2.19}$$

Both these relations are derived and discussed in the section by Kraemer in Svedberg and Pedersen (1940).

Sedimentation Technique

The technique of sedimentation as applied to viruses is quite varied. Because viruses are relatively large, their sedimentation constants are high, and elaborate long centrifugation processes are really not needed. One simple technique, used by Elford (1936), is to sediment in a capillary tube, held horizontally, and then to sample the virus at definite radii after exposure to the centrifugal action for known lengths of time at a known rate of rotation. He applied this to measure the sedimentation of bacterial viruses. The same method was used by McIntosh and Selbie (1937) and more recently, for potato yellow dwarf virus, by Brakke, Black, and Wyckoff (1951). The capillary tubes used by these workers were 2 mm in diameter, and were placed in a centrifuge cup filled with water and spun in an International 1SB centrifuge for 5 hr or so. At the end of the centrifugation, a special fine capillary tube, turned to a hook shape at the bottom, was inserted, and samples taken out at known depths. The samples were then appropriately diluted and tested for infectivity. When such a method is used, the sedimentation constant is calculated from the formula derived by Svedberg and Pedersen (1940), namely

$$S_{20} = \left\{\frac{\eta(\rho_v - \rho_0)}{t\omega^2\eta_{20}(\rho_v - \rho)}\right\}\left\{\ln\frac{x_2}{x_1}\right\} \tag{2.20}$$

where S_{20} is the sedimentation constant at 20° C, η is the viscosity of the solvent at experimental temperature, η_{20} is the viscosity at 20° C, ρ_0 is the density of water at 20° C, ρ_v is the anhydrous density of the virus, ρ is the density of the solvent at experimental temperature, t is the time of centrifugation in seconds, ω is the angular velocity in radians per second, x_1 is the

distance of the initial boundary from the axis of rotation, and x_2 is the radius of the boundary after a time t.

The sampling technique described above must be aimed at locating the position of the boundary. In place of finding the boundary at a known time, the time to reach a definite radius is measured. This is done by plotting the concentration on the upper side of x_2 at different times and by interpolating between points determining the time at which the concentration has dropped to zero.

Equation 2.20 is the product of two terms. The term $\frac{1}{t\omega^2} \ln \frac{x_2}{x_1}$ is concerned with the description of the motion of the boundary, and the term $\frac{\eta(\rho_v - \rho_0)}{\eta_{20}(\rho_v - \rho)}$ is the term which reduces the observed sedimentation to standard conditions at 20° C.

When such measurements are made, it is usually found that the value of the sedimentation constant depends on the concentration of the virus used. This is interpreted as being due to the presence of impurities which modify the rate of sedimentation by introducing local variations of density, and the normal procedure is to plot the sedimentation constant as a function of concentration and extrapolate the line back to zero concentration, where extraneous effects are assumed to be nil. It will be seen later that correction for the viscosity of the solution removes some of the dependence on concentration, a point made by Lauffer.

The availability on the market of a single wire suspension, vacuum ultracentrifuge has widened the interest in this type of research. The Spinco ultracentrifuge, manufactured by Specialized Instruments Corporation of Belmont, California, follows a design by Bauer and Pickels (1937) and is now found in many laboratories. It consists of a rotor of aluminum alloy, shaped for dynamical balance, with a hole cut through it about three inches from the axis of rotation. The hole accommodates a wedge shaped cell, with accurately ground, flat glass plates top and bottom, and within which the virus suspension can be placed. As rotation proceeds, this cell is brought, once per

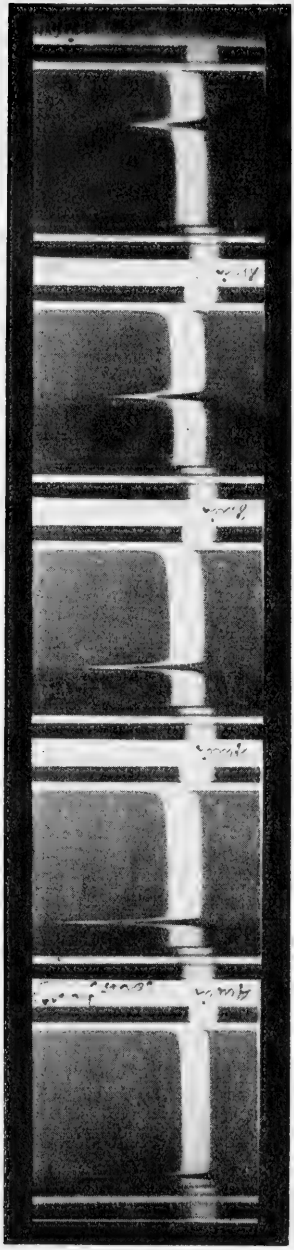


FIG. 2.4. Sedimentation diagram for southern bean mosaic virus, taken by Caspar. Sedimentation is from left to right. The virus is not quite pure and a second component can be seen.

revolution, into a beam of monochromatic light. The centrifugal action causes a virus boundary to develop, with solvent on the inside and the outward moving virus on the outside. For viruses, this soon becomes very sharp, and, at the boundary, very rapid change of refractive index occurs. The position of this boundary can be made visible by Töpler's *schlieren* method, and the optical system accordingly includes a lens, which focuses an image of the cell onto a photographic plate, and an edge across the lens. The effect of a changing refractive index is to send behind the edge a part of the cell image and, accordingly, to have a part of the image missing on the photographic plate. This can be modified by using an oblique bar and a cylindrical lens to give an up and down deflection of the trace at the boundary. Such patterns are very familiar in the literature of macromolecules today. One such is shown in Fig. 2.4, where the virus being spun is southern bean mosaic. The virus preparation can be seen to be not quite homogenous, and a filter placed across the light source produces a definite absorption at the boundary of the impurity. The main sedimentation occurs at the expected rate. This picture was taken by Mr. D. Caspar in the author's laboratory.

The speed of the ultracentrifuge is accurately controlled by a relay system, and pictures can be taken at known intervals so that the sedimentation constant can be measured directly.

A very beautiful development of the ultracentrifuge has recently been made by Beams, Ross, and Dillon (1951). If a strong magnetic field has both a radial and an axial gradient, then a rotor of ferromagnetic material will seek the region of highest field and, having found it, will remain suspended there as long as the magnetic field is present. In Beams' device, the rotor is brought up to speed, released of all material contact, and left to spin in an evacuated chamber with magnetic support. The rotor loses one revolution per second per day, and so the motion is essentially uniform. Two years would be required for the rotor to come to rest if left alone. This method is suitable for equilibrium studies in which the sedimentation drift is balanced by the tendency to diffuse. This may be of more

importance for protein studies but, as the purity of virus preparations increases, will play a part in virus research.

Diffusion-Constant Measurement During Sedimentation

A rough value for the diffusion constant of a virus can be obtained while a sedimentation run is in progress. The measurement is in terms of the gradual diffusion of the boundary. If C_z is the concentration at a distance z from the boundary, C_0 the concentration in the solution where diffusion has not yet developed, then C_z follows the form of an error integral, and

$$C_z = \frac{C_0}{2}\left\{1 - \frac{2}{\sqrt{\pi}}\int_0^y e^{-y^2}dy\right\} \tag{2.21}$$

By taking experimental values of C_z, deduced from pictures taken during centrifugation, the value of y at any point z can be found. The diffusion constant, D, is then

$$D = \frac{z^2}{4y^2t} \tag{2.22}$$

where t is time.

The above was pointed out by Svedberg, and the method is described by Svedberg and Pedersen (1940). Such a method of measuring the diffusion constant, although useful since one experiment gives two pieces of data, is not too accurate because of the short times of diffusion, the lack of temperature stability, and the wedge-shaped cell used. Nevertheless, as Lauffer (1942) has pointed out, diffusion measurements made in this way can give very good additional evidence regarding the homogeneity of the preparation.

Hydrated Partial Specific Volume

The hydrated partial specific volume can be found by sedimentation measurements in solvents of different densities. The value of ρ, the solvent density, can be varied by introducing an additional solvent until the medium reaches the condition where $\rho V_0 = 1$, when there is no sedimentation. Actually it is

not necessary to proceed to the actual point of no sedimentation, for we can rewrite Eq. 2.13 in the form

$$\eta S = \frac{m}{6\pi a}(1 - V_0\rho) \tag{2.23}$$

and by plotting ηS versus ρ there appears a straight line of descending slope with intercept on the density axis where

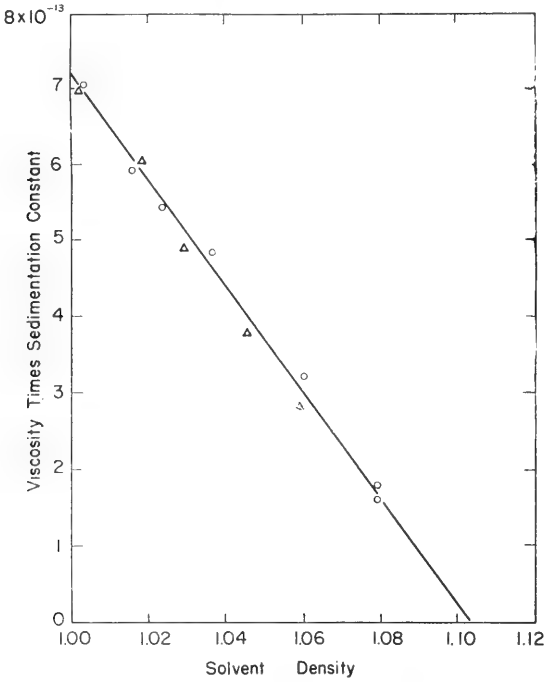


FIG. 2.5. Product of sedimentation constant and viscosity for PR 8 influenza plotted against solvent density as the concentration of serum albumin is increased, thus raising the density. The intercept value for the density determines the hydrated partial specific volume. Data due to Sharp, Taylor, McLean, Beard, and Beard (1944).

$V_0\rho = 1$. Such a line for PR-8 influenza virus, plotted from data due to Sharp, Taylor, McLean, Beard, and Beard (1944), is shown in Fig. 2.5. The additional solvent material is bovine serum albumin, which is a relatively large molecule and so does not introduce effects due to osmotic pressure. The intercept at a

density of the medium of 1.104 gives the value 0.906 for the partial specific volume.

The use of sucrose as a buoyancy variant substance has often been employed. This is a small molecule and so introduces osmotic effects. However, if the slope of the line at low concentrations is used to extrapolate to the zero-force point, the figure obtained should be valid.

For smaller, or thinner viruses, this method does not always yield consistent results, and Schachman and Lauffer (1949) have suggested that a layer of water, half the thickness of solute molecules, forms on the virus. For larger viruses this is not a very serious correction.

Before describing some actual measurements on viruses, a description of the place of studies of viscosity in virus research is in place, and then a general discussion of this approach can be given.

Viscosity

When very pure virus preparations are available, so that the virus solution can be treated as containing only virus particles and solvent, the measurement of the viscosity of the virus solution can give information regarding size and shape. The viscosity of a liquid is measured in terms of a rate of shear of the liquid, and, since a force is required to push one layer of liquid over another at a certain rate, viscosity is essentially a process in which mechanical work is continually being dissipated. The rate of such dissipation is measured by the quantity $\eta A(dv/dx)v$, where v is the velocity of the liquid, A is the area of a plane surface of the liquid, dv/dx is the rate of change of velocity with distance at the area A, and η is the coefficient of viscosity. Einstein (1906, 1911) investigated the flow of liquid around a small sphere—small, yet large compared to molecular separations—and derived an expression for the energy dissipation due to viscous flow around this sphere. He then examined the energy dissipation for a very large number of such spheres and derived the approximate expression

$$\eta/\eta_0 = 1 + 2.5\phi \qquad (2.24)$$

where ϕ is the fraction of the volume occupied by the spherical particles, and η and η_0 are the viscosities of the solution and the medium, respectively. This method, combined with diffusion, was suggested by Einstein as a method of measuring molecular radii and so of obtaining Avogadro's number. In Einstein's first paper, published when Avogadro's number was still in process of being determined, the constant 2.5 was given as unity. The resulting low values of Avogadro's number determined by this useful method were thought of as in reasonable enough agreement. The later paper corrected the figure, and accurate values of Avogadro's number resulted.

Notice that the essential character of the study of viscosity is the study of a total volume which interferes with the sliding of planes of water over one another. This total volume is, of course, the number of particles times the volume of each, but the volume concerned is basically the volume due to each particle which does not enter into slipping. So any water held by the particle is to be included in the volume ϕ. Thus viscosity measurements differ from sedimentation measurements in that, although bound water can be argued as equivalent to buoyant water for centrifugal action, and so not concerned in the motion, there is no question of buoyant action in viscosity. So one can turn a dark and a light shade of gray into black and white and assert that the particle mass determined by sedimentation and diffusion is the dry mass, while the volume determined by viscosity is the hydrated volume. This is probably true for protein molecules but is not so apt to be true where viruses of complex internal morphology are concerned. Nevertheless we are going to continue with the black and white idea because all that can be done is to try out reasonable hypotheses in the hope that a fairly large category of viruses will fit them to the first approximation. From there, as measurements improve, the second approximation can be made. So we point out the value of viscosity studies as measuring the size of a particle which fails to slip and which is, therefore, the virus plus its hydration.

There are many ways of measuring viscosity. An absolute value can be obtained by measuring streamline flow in a capillary

or by the rotation of one cylinder inside another with liquid between. Once absolute values are established, relative values can easily be found by timing the flow of a fixed volume of fluid through a certain length of capillary under the action of gravity, or by timing the rate of fall of a small sphere through the liquid. Thus viscosity is a relatively easy measurement.

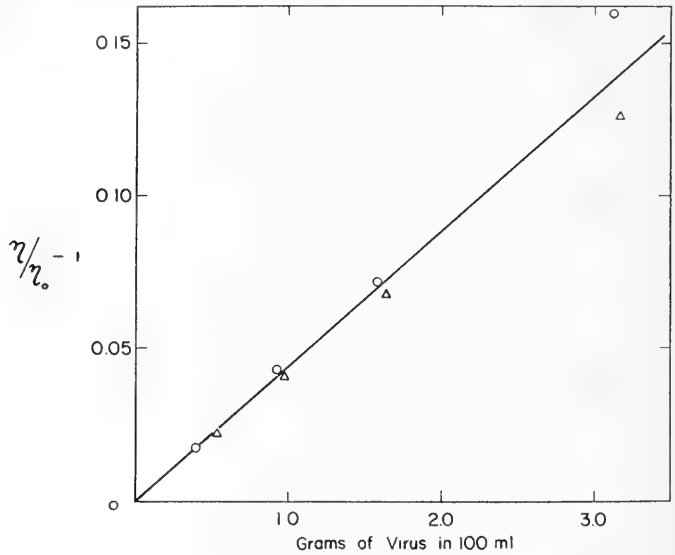


FIG. 2.6. Plot of $\eta/\eta_0 - 1$, the relative viscosity less unity, for solutions of SBMV of different concentrations. The circles and triangles refer to physically and chemically purified virus. The slope of the line enables a determination of V/m, the volume fraction of the virus, to be made. Data of Miller and Price (1944).

The Einstein equation is seen to be very powerful in the case of purified spherical viruses. The number of virus particles per milliliter can be measured in terms of the mass of dry virus per milliliter, and the mass of the virus found by sedimentation and diffusion. If C is the mass per milliliter and m is the mass of each virus particle, then the number of particles per milliliter is C/m. Now if the actual volume of each, water included, is V, we have $\phi = CV/m$, the number per milliliter times the volume of each. So a measurement of η/η_0 as a function of C tells us the

value of V/m. Now mV_0 is the actual volume of the particle, so $V - mV_0$ is the associated volume of water. Then $\frac{V - V_0 m}{m}$, or $\frac{V}{m} - V_0$, is the mass of water per gram of anhydrous virus, the conventional method of expressing degree of hydration. This ratio is denoted by r.

This method is well exemplified by the work of Miller and Price (1946) on southern bean mosaic virus. Their results are shown in Fig. 2.6. The measured values for $\frac{\eta}{\eta_0} - 1$ are plotted against C, the gram concentration of virus. The points fit a straight line quite well, so that Einstein's relation is obeyed. In these studies, the virus was purified by chemical precipitation and by ultracentrifugation, and viscosity determinations were made for each. The agreement is very satisfying.

From the slope of the line, ϕ is found to be 1.76 and so V/m is 1.76. Pycnometric measurements give the unhydrated partial specific volume as 0.70, so the mass of water per gram of anhydrous virus is found to be 1.06.

Asymmetric Particles

The pleasantness and simplicity of working with spherical particles ceases to hold if the viruses are rod shaped and becomes unmanageable if some description in terms of ellipsoids is not applicable. This doesn't mean that queer shapes do not diffuse and sediment in a regular way, but that measurements do not tell us their shape. Virus work is still at an early enough stage so that we are content to take any information and add it up. Nevertheless, the sedimentation of phage-shaped particles is not a powerful way of studying their form.

The central point to study is clearly the viscous flow of odd shapes. Neither the centrifugal reaction forces nor the "osmotic" forces which apply in sedimentation and diffusion are dependent on shape, but the viscous drag does definitely depend on shape. The problem of viscous drag experienced by ellipsoids was solved by Gans (1928). Ellipsoids permit precise solutions

of hydrodynamic equations and so can be handled mathematically. The application of these results to diffusion was made by Herzog, Illig, and Kudar (1934) and F. Perrin (1936). Their results are equivalent and lead to the expressions below. The ratio of the diffusion constant D, for an ellipsoid of revolution (semi-axis of revolution of length a, the other, b) to that, D_0, for a sphere of the same mass and volume is

$$\frac{D}{D_0} = \frac{\sqrt{1 - (b/a)^2}}{\left(\frac{b}{a}\right)^{2/3} \ln\left(\frac{1 + \sqrt{1 - (b/a)^2}}{b/a}\right)} \tag{2.25}$$

for prolate ellipsoids, and

$$\frac{D_0}{D} = \frac{\sqrt{(b/a)^2 - 1}}{\left(\frac{b}{a}\right)^{2/3} \text{arc tan } \sqrt{(b/a)^2 - 1}} \tag{2.26}$$

for oblate ellipsoids. We can consider these as measurements of the ratio f/f_0, where f and f_0 are the frictional constants for ellipsoids and spheres.

The extension of this idea to the calculation of the viscosity is much harder. Guth (1936) and Simha (1940) have derived expressions for the viscosity of solutions of prolate and oblate particles. These are not as firmly established as the Einstein equation but they represent the best available. The relations express viscosity in terms of concentration and the force constant for the particular shape, which can accordingly be found. The shape is, however, only determined as far as the ratio to spherical is concerned, and this depends on the true mass of the hydrated particle. There is thus no clear separation of hydration and shape.

Now, in the measurement of f from diffusion studies, it will be remembered that the viscous-drag term corresponds to whatever actual particle is being pushed through the medium. If some water is bound to the particle and changes its radius or surface, this must be included. So the measurement of f by the diffusion measurement will not yield b/a unless the proper hydrated

particle is being treated. Thus, again, an ambiguity between hydration and axial ratio exists.

What is done is to assume a reasonable hydration and axial ratio to fit diffusion data and then to see whether a check against viscosity is obtained. Actually, there is no virus for which the whole elaborate technique of protein molecular measurement has been applied. Very nearly, it has been done for tobacco mosaic virus and southern bean mosaic virus, with results to be described in what follows.

Examples of the Use of Virus Motion Studies

Some examples of virus motion studies can be given here. These are by no means the only cases studied, but are chosen as representative of the kind of work that can be done. The first is the study of potato yellow dwarf virus by Brakke, Black, and Wyckoff (1951). The purification of this virus to a high degree is difficult, as it is not very stable and centrifugation has to be carried out near the freezing point. Nevertheless, concentrated preparations of high infectivity could be obtained, and these were centrifuged in horizontal capillaries. During the centrifugation a quite definite visible boundary developed, and it was possible to measure the sedimentation constant of this boundary. In addition, virus samples were removed by a fine capillary, and the sedimentation constant of the infectivity was determined. The constant varied with concentration. This was traced to the fact that the viscosity of the solution increased with concentration. By plotting the apparent sedimentation constant versus concentration, a reasonably good straight line was obtained which was extrapolated back to zero concentration to give a value of 1,150 S for water at 20° C.

The variation of sedimentation rate with solvent density was plotted, using sucrose as the second solute. The results extrapolate to a zero sedimentation rate at a density of 1.17, so the hydrated density of the virus is 1.17. This may need correction for the osmotic effects of the small second solute molecule.

The authors examined, with some care, several high concentration preparations of potato yellow dwarf virus in the electron

microscope. The field contained particles of rather irregular circular shape, with estimated volumes spreading from 5.0×10^8 Å^3 to 1.3×10^9 Å^3. The mean volume was close to 8.5×10^8 Å^3.

If the electron micrographs are taken as justification for treating the virus as approximately spherical, the authors deduce, from Eq. 2.13 for sedimentation, that the virus diameter is 1,100 Å, the volume is 6.9×10^8 Å^3, and the "molecular weight" is 490,000,000. The hydrated density of 1.17 is relatively low and implies a high degree of hydration, which may partly account for the virus instability.

The value of the above studies lies mostly in the fact that the correlation between the objects seen in the electron micrographs and the rate of sedimentation of infectivity is very good, so that it can be concluded that the electron microscope observations are not merely of plant debris. The electron microscopy thus acquires added strength, and a further proposal that possibly the virus particles are more ellipsoidal in character can be made.

It is of interest that the visible boundary sedimented at the same rate, within experimental error, as the infectivity. This offers some more evidence that the virus particles are indeed the infectious bodies, but only circumstantially so.

The above study is chosen because it may well be representative of virus work. Viruses, in general, are not exceptionally stable, can not be highly purified, and are hydrated so much that simple physical observations on them are difficult. The conclusions that can be drawn are nevertheless not trivial, they serve to characterize the virus particle as a definite object and are helpful in further studies.

It is to be regretted that very few studies of the rate of sedimentation of plant viruses as measured by serological affinity have been made. This virus assay is rather easier than the local-lesion technique, and the results would add a further piece of information regarding virus size and shape.

As a second example we can choose southern bean mosaic virus, studied by Miller and Price (1946). This is a spherical plant virus capable of being highly purified, and so is fully

suitable for detailed study. The preparations used were not crystalline but were purified by both sedimentation and chemical methods. Both processes gave the same results. Sedimentation measurements were made at various concentrations. The sedimentation constant slowly fell as the concentration increased. By plotting $1/S$ versus concentration and extrapolating the resulting straight line to the value $1/S$ at zero concentration, the sedimentation constant at 20° C was determined to be 115 S.

Diffusion measurements were carried out in an electrophoresis cell, with the result that the diffusion constant at 20° C was found to be 13.4×10^{-8} cm^2/sec. Partial-specific-volume measurements on the dry virus gave the value 0.696 ml/gm. Putting these together, the particle molecular weight was found to be 6.63×10^6.

The individual sedimentation and diffusion measurements were then applied to Eq. 2.17 for the frictional drag ratio, with the result that a value of $f/f_0 = 1.25$ was found.

The viscosity of the virus was measured as a function of concentration, with the results shown in Fig. 2.6. The value of ϕ so found was 1.76 if the particles are assumed to be spherical and the Einstein relation, $\eta/\eta_0 - 1 = 2.5\phi$, can be applied. Alternatively, if the virus is supposed to be unhydrated, and an axial ratio is responsible for the large viscosity, the axial ratio deduced is 5.5 to 1.

In addition to these measurements, the sedimentation rate in sucrose solutions of different density was measured. The sedimentation rate did not vary linearly with density, probably due to osmotic effects or combination between virus and sucrose. However, by carefully determining the slope of the line at very low sucrose concentrations and extending this to the point of zero net force, the density value for the sucrose solution was found to be 1.21, corresponding to a partial specific volume (hydrated) of 0.827. The curvature of the line was actually slight, though definite, and the data are so good that the above figures should be very reliable.

We now have the following experimental facts about the virus. First, the particle molecular weight is 6.63×10^6. Second,

diffusion measurements give f/f_0, the frictional ratio, as 1.25. Third, viscosity measurements give ϕ, the apparent volume fraction, as 1.76. Fourth, the dry partial specific volume is 0.696 gm/ml, and the hydrated partial specific volume is 0.827.

These can be explained in only one way. If we suppose that the f/f_0 value from diffusion is due to unhydrated ellipsoidal particles, then the axial ratio deduced from Eq. 2.25 is $a/b = 5$. This agrees with the value found from viscosity measurements. However, the hydrated partial specific volume figure does not fit this idea. Lansing and Kraemer (1936) have shown that if V_0 is the partial specific volume of a combination of two substances with separate values V_D and V_1, and if r grams of substance V_1 combine with 1 of substance V_D, then

$$(r + 1)V_0 = rV_1 + V_D \tag{2.27}$$

Putting in the above figures, we find $r = 0.76$ gm of water per gram of dry virus.

Now this radically changes the explanation of both the diffusion and the viscosity figures. In the former case, if we assume spherical viruses and apply Eq. 2.18 for f/f_0 as due to hydration, the value of r so found is 0.78 gm water per gram of virus. This agrees beautifully with the direct measurements. Using the value of $V/m = 1.76$, together with the dry partial specific volume of 0.696, the relation $r = V/m - V_0$ gives $r = 1.06$. This is rather high, but still in tolerable agreement. Miller and Price then conclude that an average value of 0.83 gm of water of hydration is associated with each gram of virus; that the virus is spherical; and that its mass, independent of hydration, is 1.1×10^{-17} gm, corresponding to a particle molecular weight of 6.63×10^6. The hydrated particle has a diameter of 312 Å, and the dry particle a diameter of 244 Å.

Very recently, Lauffer, Taylor, and Wunder (1952) have made quite extensive studies on the centrifugation of southern bean mosaic virus. They have examined very carefully the sedimentation in the presence of bovine serum albumin and various alkali metal chlorides and also at different pH values and in sucrose. Their studies show that buoyancy methods can readily be sub-

ject to systematic error and that several factors need to be considered before specific volumes are deduced. They conclude that rather less water is associated with the virus, giving the value 0.45 gm water per gram of virus. In consequence, the virus diameter is also smaller, being 280 Å.

It must be agreed that, used in this way, virus motion studies are highly informative.

As a third illustration of virus motion studies we can consider tobacco mosaic virus, as described in papers by Lauffer (1944) and Schachman and Lauffer (1949). All the beauty and simplicity of the study of spherical particles are here absent. Tobacco mosaic virus, although it can be thoroughly purified, has a long rod structure which readily aggregates into multiple lengths. Thus sedimentation, diffusion, and viscosity measurements are all subject to the question of whether the virus has aggregated or not. Nevertheless, Lauffer carried out sedimentation measurements on the preparation showing the least viscosity. In the course of these measurements he was able to show that the dependence of sedimentation constant on concentration is due to a single factor, the viscosity of the solution in which the virus is moving. In Fig. 2.7 is shown a plot of Lauffer's data before and after the correction for the solution viscosity. The apparent sedimentation constant is plotted as a function of virus concentration, and it can be seen that the use of solution viscosity (which is changing markedly with tobacco mosaic concentration) in place of solvent viscosity produces a reasonably constant set of values. The value found for S_{20} was 187 S. This treatment was also found to be valid for SBMV by Miller and Price.

For this same preparation, the diffusion constant was found to be 2.62×10^{-8} cm^2/sec, and the unhydrated partial specific volume to be 0.73 ml/gm. Using these figures, a particle mass of 5.24×10^{-17} gm, or a particle molecular weight of 31.6×10^6, can be calculated. There now arises the same question of axial ratio and hydration. Specific viscosity measurements combined with sedimentation measurements yield an axial ratio of about 20. However, hydration must play a part. To establish this,

Schachman and Lauffer made sedimentation rate measurements in both serum albumin and sucrose solutions, with the results shown in Fig. 2.8. It can be seen that the no-net-force point occurs for a solvent density of 1.127 in the first case and 1.266 in the second. These do not agree. The first requires hydration to the extent of 66% by volume per milliliter of virus, and the

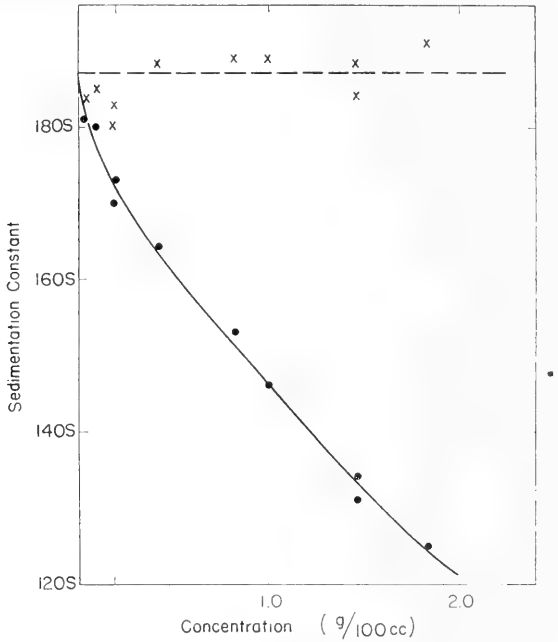


FIG. 2.7. Sedimentation constant of TMV versus concentration. The viscosity of the solution increases as the concentration increases and, if allowance is made for this, the dotted line results. Data and analysis of Lauffer (1944).

second to 27%. Following a suggestion made by Kauzmann, they propose that a layer of water roughly half the thickness of the solute molecules is entrained by the virus. Correcting for this, they conclude that the hydration of tobacco mosaic virus is 15% of the wet particle, or about 0.12 gm water per gram virus. It is likely that the hydration of SBMV is less if this type of correction is made.

With this figure, the sedimentation, diffusion, and viscosity data fit with a rod-shaped virus of length 2,500 Å and width

140 Å. It is truly remarkable that so difficult a virus to study should nevertheless be susceptible to correct characterization by these means.

The fourth illustration is the study of rabbit papilloma virus carried out by Neurath, Cooper, Sharp, Taylor, Beard, and

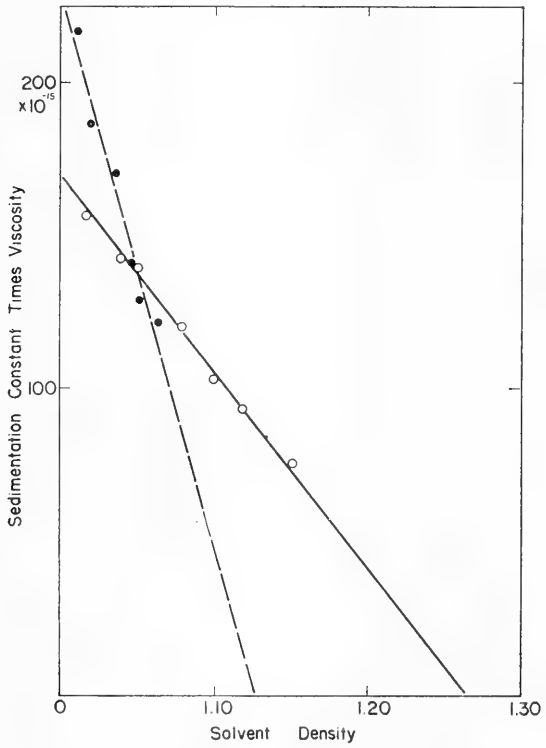


FIG. 2.8. Buoyancy measurements by Schachman and Lauffer (1949) on TMV using two second solutes. The steep slope is for serum albumin, the gentle slope for sucrose. Differential entrainment of water is suggested to explain the two slopes.

Beard (1941) and Sharp, Taylor, Beard, and Beard (1942). Rabbit papilloma virus can be obtained from warts in Western cottontail rabbits. The collection of material from four trapping seasons gave 430 gm of wart tissue. This was ground and extracted, and the virus separated by multiple centrifugation.

Since the wart tissues were stored at 2–5° C for several years, it is obvious that this is a stable virus.

Sedimentation measurements gave a value of $S_{20} = 278$ S. Diffusion measurements by the refractometric method gave D_{20} as 5.85×10^{-8} cm^2/sec, and the anhydrous partial specific volume was found to be 0.754. These, when put together, give the particle mass as 7.81×10^{-17} gm and the particle molecular weight as 47×10^6.

Putting together the sedimentation and diffusion data to calculate f/f_0, the value obtained is 1.486, which is rather high. If an unhydrated virus is assumed, the axial ratio deduced is 9 for a prolate ellipsoid and 11 for an oblate ellipsoid. However, there is no reason to assume an unhydrated virus, and if, instead, a spherical virus is assumed, the application of Eq. 2.18 gives 1.73 for r.

In addition to sedimentation and diffusion measurements, the viscosity at various concentrations was observed. The data are very good and show a straight line with a slope of 0.063 for $\eta/\eta_0 - 1$ plotted against gram-percent concentration of virus. The value of V/m is, accordingly, 2.52, and the resulting hydration is 1.77 gm water per gram dry virus. This agrees well with the value above. Subsequent observation of the virus in the electron microscope showed a spherical particle of diameter 420 Å, which fits the molecular weight of 47×10^6 reasonably well, if the partial specific volume of the dry virus as measured is used.

The conclusion seems to be, therefore, that, in solution, rabbit papilloma virus is considerably hydrated but is still spherical.

Lauffer and Stanley (1944) describe electron micrographs, sedimentation, specific volume, buoyancy, and viscosity measurements on PR8 influenza virus. The electron micrographs give a diameter of 1,150 Å and an apparently spherical shape. The specific volume of the dry virus was 0.79. The density of sucrose for flotation was 1.18, so that the wet density is 1.18. The viscosity measurements did not show a linear increase in relative viscosity with virus concentration, a fact probably due to the presence of nonvirus components in the preparation. From these

measurements, Lauffer and Stanley conclude that the particle diameter is about 1,000 Å and the degree of hydration is 0.60 gm of water per gram of virus. Lauffer and Taylor (1953), with more modern buoyancy observations, find the sedimentation constant for the virus to be slightly higher and, from the two sets of data, conclude that the hydration is 0.32 gm water per gram of virus.

It is hardly possible to tabulate here all the observations on virus motion studies. Bawden (1950, p. 224) tabulates the sedimentation constants of 11 plant viruses, and Lauffer, Price, and Petre (1949, p. 188) tabulate sedimentation constants for a variety of viruses, and diffusion constants for four viruses.

Two cases are of some interest. The first is turnip yellow mosaic virus. This has been studied by Markham and Smith (1949). Preparations of this virus can be separated into two layers in the ultracentrifuge. The upper layer contains no nucleic acid and is not infectious, whereas the lower, denser layer is nucleoprotein, which is infectious. The serological properties of the two layers are the same, and the diffusion constant of the upper layer is 1.51×10^{-1} cm^2/sec, of the lower 1.55×10^{-1}. So the particle size is slightly different. Yet the sedimentation constant for the lower layer is 106 *S*, and for the upper layer it is 50 *S*. There is, therefore, a considerable difference in virus mass. This can, in part, be related to the nucleic acid. X-ray studies described in the next section indicate that, in addition, there is a higher density.

A second case of interest is the Rothamsted variety of tobacco necrosis virus for which Ogston (1942) finds two sedimentation constants of 240 and 51 *S*. Bawden and Nixon (1951) report that these are two spherical components of diameter 370 and 180 Å.

In comment on this work it should be pointed out that the basic physical methods of size and shape measurement by motion studies are vulnerable to errors of interpretation. It has already been seen that hydration and axial ratio can be confused. It is quite likely that a reconsideration of the actual physical factors involved may change detailed points of the measurement analysis. Thus the recent theory of Kirkwood and Shumaker (1952)

may modify conclusions regarding protein molecules. These modifications may extend into virus figures, although, in view of the excellent check given by the electron microscope, this is not likely.

X-Ray Diffraction Applied to Viruses

X-ray diffraction is a powerful physical tool which is being applied to supply evidence regarding crystalline protein structure. For diffraction studies to yield really valuable information, relatively large, single crystals of material are needed. In any event, some order in the molecular arrangement is necessary. Only for some preparations of plant viruses has it been possible to apply this method.

X-ray diffraction is concerned with three classes of measurement. The first concerns the regular atomic arrangements inside each virus particle. The second concerns the size of each particle, which appears as a repetitive diffracting distance. The third concerns the space between viruses, which may be a very regularly repeating value. All three types of diffraction have been observed. The first two are of use in describing particle size and shape and will be treated here. The intervirus spacing is of more interest later on and will be deferred until then.

Diffraction arises from phase-related scattering. It is simplest to visualize this as due to regularly repeated scattering units, which will cause diffraction maxima at certain definite angles which are related to the repetition distance. The best illustration of this is the reflection from planes rich in whole virus particles. A very beautiful picture of the nature of a small crystal of tobacco necrosis virus, taken with the electron microscope by Wyckoff, is shown in Fig. 2.9. It can be seen that there are regular, closely populated planes, and indeed it can be seen how the edges of the crystal are formed. Now any closely populated plane is one which scatters X-rays richly. Bragg pointed out that from such a plane equal angles of incidence and scattering give a diffraction maximum. If, in addition, there are many parallel planes, certain definite angles are selected, and these angles are related to the X-ray wavelength, λ, the separation of the richly

populated planes, d, and the angle of scattering, θ, by the Bragg relation $n\lambda = 2d \sin \theta$, where n is an integer.

An analysis of such crystals of tobacco necrosis virus was made by Crowfoot and Schmidt (1945). The crystals used had been grown for more than a year, and the largest was $2 \times 1 \times 0.5$ mm. Both still and oscillation pictures were taken, with the

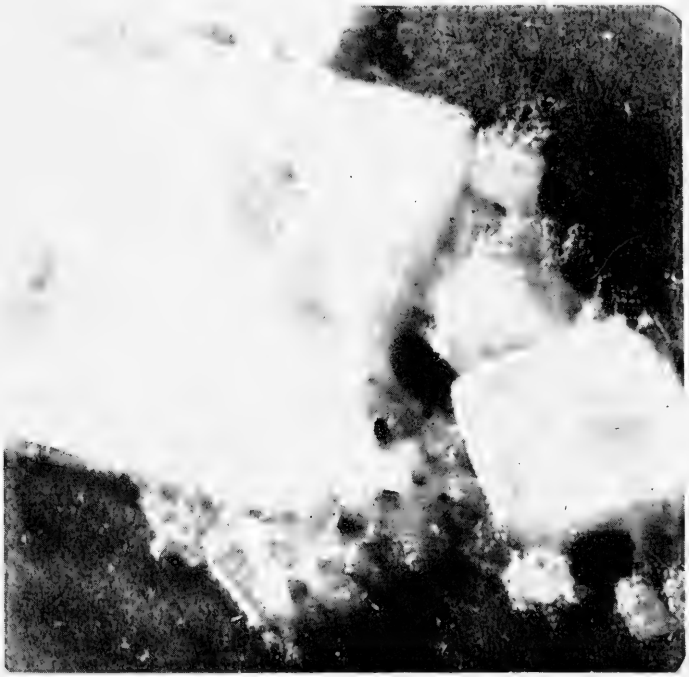


FIG. 2.9. Electron micrograph of a small crystal of tobacco necrosis virus, taken by Wyckoff. Reprinted from Electron Microscopy by R. W. G. Wyckoff. Copyright 1949, Interscience Publishers, New York, London.

result that the basic pattern corresponds to the packing in contact of a set of spheres of diameter 160 Å. It is remarkable that over 500 hr of X-ray exposure could be given this crystal without any apparent change in its crystalline structure. This has an important bearing on other results involving radiation sensitivity. The great accuracy of more usual crystal analysis by X-rays is not present here since the crystalline perfection of chemical crystals is not matched by the virus crystals. With

still smaller crystalline preparations, Bernal, Fankuchen, and Riley (1938) found a spherical particle diameter of 270 Å for bushy stunt virus.

If in these crystals each virus could be identically oriented, it would be possible to find out some information about the internal atomic spacing. For spherical viruses this has not been done, but for tobacco mosaic virus, the long rod shape does frequently cause rather accurate mutual orientation. Using such preparations, Bernal and Fankuchen (1941) found that there is an

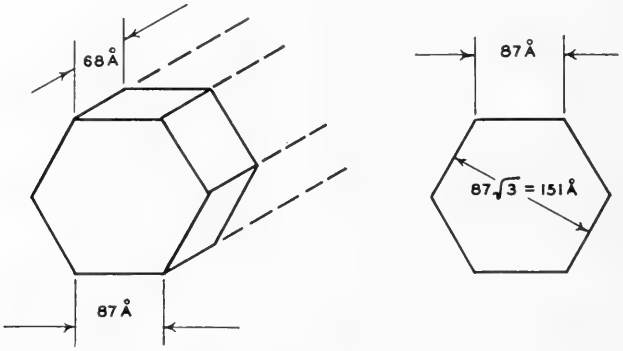


Fig. 2.10. Unit cell of tobacco mosaic virus as determined by Bernal and Fankuchen (1941).

internal structure with a hexagonal lattice. To make their data fit this lattice, they found it necessary to use some fractional values of n in the Bragg formula, where n is supposedly integral. Bernal and Fankuchen point out that in a virus there is nowhere near an infinite atomic array. Hence the atomic planes do not select precisely integral values for scattering. If this interpretation of their data is right, the unit cell they find is as shown in Fig. 2.10. The hexagon side is 87 Å long, and the cell depth is 68 Å. It is very gratifying that the precision electron microscopy applied by Williams (1952) to this virus also shows a hexagonal cross section of the same size. Thus Bernal and Fankuchen's unit cell has one cross section which is that of the virus itself.

The same method has been applied to tomato aucuba mosaic virus, enation mosaic virus, and cucumber viruses 3 and 4, all of which are related to tobacco mosaic virus. The same width

of 151 Å was found for all, except the cucumber viruses, which had a somewhat smaller width of 146 Å.

Bernal and Carlisle (1948) have made most interesting studies on turnip yellow mosaic virus. This virus is remarkable in that, as has been previously mentioned, the crystalline preparations separate ultracentrifugally into two factions, one contains nucleic acid and is infectious, and one has no nuclei acid and is non-infectious but has the virus serological properties. Bernal and Carlisle found that both classes of crystalline preparations had the same kind of X-ray structure, but that the virus repeat dimension for the infectious type was 228 Å, whereas for the non-infectious, nucleic-acid-free particles it was 238 Å, or 10 Å bigger. This remarkable fact suggests to Bernal and Carlisle that the nucleic acid holds the protein in a more tightly bound configuration. On drying, the interparticle distance shrinks and is the figure quoted above. When wet, the distance increases by 77 Å or so.

Very interesting interparticle regularities were also observed for TMV and will be discussed later.

In the case of tobacco mosaic virus, Bernal and Fankuchen were able to secure sufficiently accurate orientation to observe reflections which correspond to some structure inside the unit cell. The difficulties of analyzing such reflections are very great, but they propose that in the hexagonal, unit cell there are platelets of dimensions $44 \times 44 \times 22$ Å which are arranged in regular order throughout the cell. The possibility of this type of analysis of internal structure makes further X-ray diffraction work on viruses of great importance.

These X-ray studies can also be used to measure hydration, or at any event to act as a check on proposed values of hydration. If observations on spherical viruses, or oriented, long, thin viruses, are made, the interparticle distance is found to be a function of concentration. In the case of TMV it was found that, as the virus was suspended in higher and higher concentrations of ammonium sulphate, the interparticle distance fell to a value of 178 Å. In the dry gel preparations, in which the virus dries in an oriented way, the separation was found to be 151 Å,

which is the separation for hexagonal rods in contact. Thus the effect of removing the dryable hydration cannot well be more than that produced in going from 178 Å to 151 Å. So extreme hydration proposals are not likely to be true. Actually, the values already quoted, as proposed by Schachman and Lauffer, fit these figures quite well.

The shrinkage of bushy stunt virus on drying reduced the interparticle distance from 394 Å to 318 Å, and the process was reversible. Thus rather higher hydration is possible.

Small-Angle X-Ray Scattering by Viruses

A method which promises to be very powerful has recently been applied to virus study by Kratky (1948) and by Ritland, Kaesberg, and Beeman (1950). The apparatus used by these last authors is shown schematically in Fig. 2.11. The virus solution is placed in a Lucite sample holder with properly oriented, thin polyethylene windows. The source of X-rays is a high-power, rotating-anode tube operating at about 30 kv and 100 ma, with a copper anode and a thin metal window. The X-rays are filtered through a nickel-cobalt filter and accurately collimated with

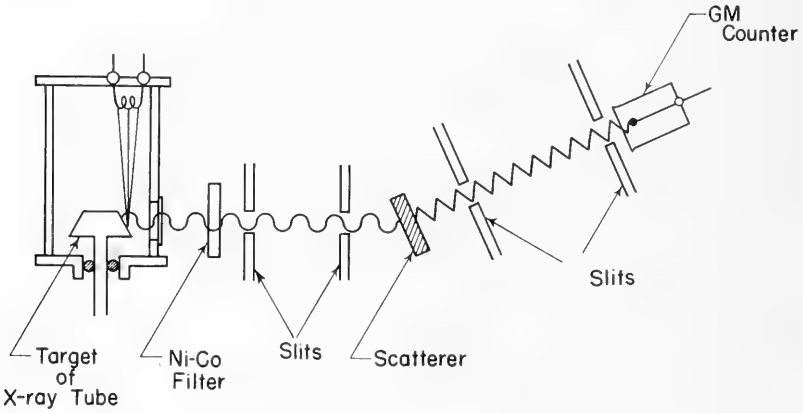


Fig. 2.11. Schematic arrangement for low-angle scattering of X-rays by viruses as used by Kaesberg, Ritland, and Beeman. A high-current rotating-anode X-ray tube supplies a beam which is filtered and collimated before encountering the scatterer. This is a solution of virus. Scattering is measured as a function of angle with the Geiger-Müller counter.

fine slits. The scattered beam is observed through two slits which can be rotated around the scatterer. The whole equipment is about 6 ft in dimensions.

Two theoretical features of the experiment need to be considered. At very small angles of scattering, ϕ, the theoretical scattered intensity is $I(\phi)$, where

$$I(\phi) = Nn^2 I_e \, e^{-\frac{4\pi^2 R^2 \phi^2}{3\lambda^2}} \tag{2.28}$$

In this expression, N is the total number of particles irradiated, n is the number of electrons per particle, R is the "radius of gyration"* of the electrons in the particle, λ is the X-ray wavelength, and I_e is the normal scattering by a free electron. This theory has been developed by Guinier (1939). A measurement of $I(\phi)$, therefore, gives the value of R^2. Note that this concerns the *electron* distribution. Hence if there is any curious structural distribution of phosphorus, a high-electron element, and hydrogen, a low-electron element, this should show up in the radius-of-gyration measurements. So far this feature has not been exploited, but it has promise.

The second theoretical point involves larger angles, for which interference from the various internal sections of the virus plays a part. If the virus is spherical, the intensity has maxima and minima given by the expression

$$I(\theta) = \text{const} \times I_0 \left\{ \frac{\sin\left(\frac{4\pi D \sin\theta}{2\lambda}\right) - \frac{4\pi D \sin\theta}{2\lambda} \cos\left(\frac{4\pi D \sin\theta}{2\lambda}\right)}{\left(\frac{4\pi D \sin\theta}{2\lambda}\right)^3} \right\}^2 \tag{2.29}$$

where I_0 is the intensity at zero angle, and D is the diameter of the virus particle θ is the half-angle of scattering. A more elaborate expression can be derived for spheroids.

One very important feature of this type of scattering experiment is that the essential scattering element is the electron distribution, which differs from water. Thus any hydration

* This "radius of gyration" is taken about the center and not about an axis of rotation. It is defined as $R^2 = \Sigma\rho dv r^2 / \Sigma\rho dv$, where ρ is density, v is volume, and r is radius, and so is formally like the mechanical radius of gyration.

which merely consists of water bound on the outside, or even mechanically seeped into cracks in the structure, will not contribute to the scattering. On the other hand, water, which causes a swelling of the whole virus, will produce an effect because the radius of gyration will be altered. Thus X-ray scattering offers a new measurement of size and shape, with a different response to water associated with the virus. More detailed information regarding hydration can thus be obtained for a virus which has been studied by sedimentation, diffusion, and viscosity, as well as by X-ray scattering.

The price that has to be paid is the need for very pure preparations of high concentration, concentrations of the order of 1% are needed, and any considerable amount of virus impurity is serious. For this reason, attractive studies of serological precipitates are not so easy to carry out.

The small-angle scattering alone yields the value of R, the radius of gyration. If the molecular weight is independently known, it is possible to calculate the axial ratio for an assumed ellipsoid. This axial ratio is for the hydration-free molecule since the effect of water is deducted from the virus. By comparing the X-ray figures with axial ratios derived from the frictional ratio in sedimentation, which corresponds to the hydrated molecule, the degree of hydration can be inferred.

A study of the two spherical viruses, southern bean mosaic and tobacco necrosis, by Leonard, Anderegg, Kaesberg, Schulman, and Beeman (1950, 1951) has enabled them to make measurements of the radius of gyration in each case using only the small angle scattering. The results are listed in Table 2.4. The interference radius of bushy stunt is included.

TABLE 2.4

Virus	Radius of gyration (Å)	Corresponding virus radius	Radius from interference maxima
Southern bean	111	143	149 ± 3.3
Tobacco necrosis	119	154	150 ± 2.8
Bushy stunt			160 ± 10

The internal interference scattering expression should apply to these viruses, and it has been found to hold. The results for southern bean mosaic virus are shown in Fig. 2.12, where it can be seen that the first five secondary maxima are visible. This is a very remarkable and most interesting result, for it shows that the conditions for large-angle scattering are closely fulfilled. Put in

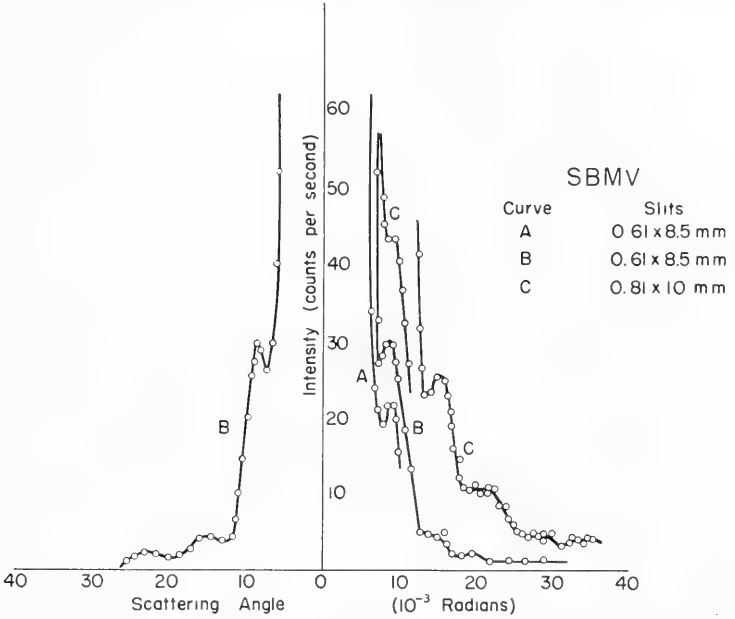


FIG. 2.12. X-ray scattering of SBMV taken by Ritland, Kaesberg, and Beeman (1950). Three sets of slits were used, and it can be seen that five sets of maxima are discernible. This means the virus is spherical to a close approximation.

simple terms: because the virus particles are not arranged in any way, their perfect symmetry about the X-ray beam must be due to their own simple shape, and, in fact, the only shape simple enough is a sphere, and an accurate sphere to boot. The experiments show that the viruses are spherical to one part in 50, which is a remarkably accurate geometrical shape. They fully justify the use of equations for spherical particles in sedimentation, diffusion, and viscosity. Similar results on tobacco necrosis virus and bushy stunt virus have been reported, so that one class of

plant viruses, at least, is spherical, and so offers a simple geometrical shape for later speculation.

This class of study adds one other datum to virus knowledge. Because any water attached externally will scatter like the water solvent, it cannot contribute to the actual net scattering. So any evidence for hydration cannot be due to water found on the surface. Now Miller and Price (1946), from sedimentation and diffusion measurements, found the value of the unhydrated virus volume to be 7.66×10^{-18} cm^3, whereas these X-ray experiments give 13.8×10^{-18} cm^3 for the volume of the internally hydrated virus. There is, therefore, 6.1×10^{-18} cm^3, or roughly 6.1×10^{-18} gm, of water for each 1.10×10^{-17} gm of unhydrated virus, which amounts to 0.55 gm water per gram virus. When this is compared with the total hydration of 0.67 gm water per gram virus, there is a residue of 0.12 gm water per gram virus, which is the external layer. This amounts to a thickness of about 4 Å and corresponds very nearly to a monomolecular layer. The same considerations can be applied to tomato bushy stunt virus with rather less accuracy. The internal hydration is found to be 0.50 gm water per gram of dry virus.

Picture of a Virus

One of the potential contributions of physical studies of viruses is some sort of pictorial representation of a virus. To some extent this can be begun as a result of the work described in this chapter. Taking as an example two of the best studied plant viruses, we deduce the appearances shown in Fig. 2.13. These are the bare canvases on which the real, detailed structure of the virus must be filled in. As further physical studies are elaborated, it will be seen that the open circle and long rod develop surface structures and internal constitutions. It should also be seen that it is far from hopeless to add a great deal of detail by patience and care.

Identity of Physical Particle and Infectious Unit

If the objects which are under study by these means are to be described in as detailed a way as we hope, it is plain that we

should be certain that we are really studying the infectious unit responsible for the expected symptoms and not something which separates from highly infectious preparations and is only circumstantially related to the actual infectivity. There is a great deal of nonphysical evidence that crystalline, plant virus

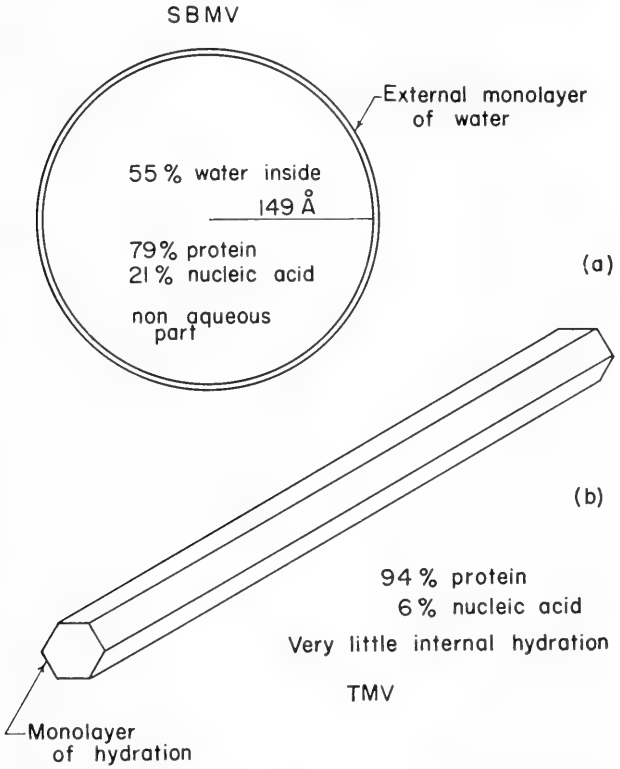


FIG. 2.13. Shape information regarding southern bean mosaic virus and tobacco mosaic virus as obtained from the physical studies described in this chapter.

preparations have all the expected infective properties. These are briefly summarized by Bawden in his book, where he points out that the purified crystalline bodies, having recognizable chemical properties which can be extracted from infected plants, are never found in healthy plants. Moreover, when different kinds of plants are susceptible to the same virus, the same crys-

talline bodies can be found in the different plants which, while in the healthy state, have no common substances which are comparable. The infectivity of purified preparations is very high and, if allowance is made for the expected loss of activity in purification, is high enough to account for all the infective agent being in the purified preparation. Finally, the antibodies produced by clarified plant sap from infected plants combine specifically and strongly with the purified virus preparations. All these facts add up to make it most likely that the preparations we call virus preparations are, in reality, strong concentrations of the infectious principle, as they should be.

A little extra confidence in this belief can be obtained by subjecting a virus preparation to ultracentrifugation and seeing whether the optical boundary of the concentrated nucleoprotein and the rate of transfer of infectivity coincide. By using a separation cell, in which a barrier across a sedimentation cell permits sedimentation through it under high acceleration, but does not allow rapid back diffusion, the rate of transfer of infectivity can be measured and compared with the rate of sedimentation by conventional means. This method has been exploited by Lauffer in particular. For three viruses, tobacco mosaic (Lauffer, 1942), influenza (Lauffer and Miller, 1944), and southern bean mosaic (Epstein and Lauffer, 1952), the infectivity follows the optical sedimentation. The identity of the two principles is therefore the more likely.

The diffusion measurements of Polson and Shepard (1949) on T-3 and T-4 bacteriophages showed, at high concentration, that the diffusion constant was in reasonable agreement with the shape of the particles seen in electron micrographs. This argues that the phage particle is indeed the infectious unit. The fact that electron micrographs of bursting bacteria show about the right population of sperm-like objects is further evidence. However, it should be realized that, whereas the virus may have one shape and structure between residences in a host, the whole of this structure may not be needed for multiplication inside the host. So there is still left a question as to the nature of the object on which the biophysicist should concentrate his attention when making theoretical speculations regarding virus multiplication.

Virus Dimensions

We conclude with a table of virus dimensions which is probably nowhere near complete but which represents some of the available information on viruses. At this stage it is worth while to see what kind of classification of viruses by shape is possible. One fact is readily apparent—many viruses are spherical. To

TABLE 2.5

Virus	Dimensions (Å)	References*
Psittacosis	4,500 diameter	
Vaccinia	2,600 × 2,100	
Herpes simplex	1,500 diameter	
Rabies	1,250 diameter	
Influenza	1,150 diameter	
Newcastle	1,150 diameter	
Staphylococcus phage	1,000 diameter head 2,000 long tail	
T-2 *coli* phage	600 × 800 head 1,500 long tail	
M-5 *megaterium* phage	760 diameter head 3,000 long tail	Friedman and Franklin (1953)
T-1 *coli* phage	500 head, 1,500 tail	Fluke (1953)
Rabbit papilloma	440 diameter	
Tobacco mosaic	2,800 long; hexagonal cross section, side 87 Å	Williams (1952)
Southern bean	298 diameter	Ritland, Kaesberg, and Beeman (1950)
Tobacco necrosis	300 diameter	Ritland, Kaesberg, and Beeman (1950)
Bushy stunt	300 diameter	Ritland, Kaesberg, and Beeman (1950)
Lansing polio	250 diameter	
Coxsackie Texas 1	340 diameter	Melnick, Rhian, Warren, and Breeze (1951)
Yellow fever	220 diameter	
Louping ill	190 diameter	
Tobacco ring spot	190 diameter	
Japanese B encephalitis	180 diameter	
Alfalfa mosaic	170 diameter	
Foot and mouth disease	100 diameter	

* The authority for a large part of this table is from Stanley's review article in Chemical and Engineering News (1947). Other references are specifically given.

some extent this is due to first-order examination of dried viruses in electron micrographs, wherein a roughly circular appearance is classed as spherical. However, the X-ray scattering studies described in this chapter show that, for three plant viruses in solution, reasonably accurate spherical shapes are found. This may well be true of many others.

There are some long, rod-shaped viruses, but these are only found among plant viruses. Among bacterial viruses, a combination of rod and sphere seems to be a plausible description, although as careful studies are made, the heads appear to be only very roughly spherical. In addition, the tails do not appear as definite rods but seem to be rather variable both in length and diameter.

These physical shapes at once pose a sharp challenge to the physicist. Virus can make more virus out of the host. Presumably, to some extent, the formation of duplicates is by physical forces—Van der Waals, valence, or electroionic. How does a spherical object so influence its surroundings as to generate a second spherical object? And if it doesn't directly do so, how is its structure broken down for multiplication so that many others can be assembled as spheres from the parts? Physics and physical chemistry has not yet encountered this type of crystallization process in which a number of large atomic aggregates of identical size and complex composition are formed from the medium. The temptation is to say that virus multiplication *must* consist of rods or plates generating other rods or plates, using the short range forces we already know. But in spite of this "must," nature efficiently produces spherical viruses. It would seem that some extra process must be present. However, it is unwise to invoke such a process until it is clear that the actual multiplicative unit is indeed the spherical virus and not some quite different sub-unit, suitably shaped for the action of physical forces. Research is rapidly bearing down on this most important question.

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CHAPTER THREE

IONIZING RADIATION AND VIRUSES

Ionization is, to a very large extent, caused by the passage of very fast, charged particles through matter. These particles have atomic dimensions or less (*much* less in the case of fast protons, deuterons, or alpha particles), and because of their high speed they are relatively unaffected by the vast majority of the atoms near their path. Thus these fast charged particles can readily penetrate into the interior of viruses, and may indeed produce no effect at all until some internal action is produced. For this reason, fast, charged particles are probes of virus structure. In addition they produce transient excitation effects in the solvent, which carry energy and can produce action on the large molecules of biology. The study of this should one day add still more to the knowledge of virus structure. The importance of studying the effect of ionizing radiation on viruses therefore lies in the fact that it is concerned mainly with the internal organization of the virus, something which is not measured by the techniques of the last chapter.

The basic idea underlying the use of ionizing radiation is that it is a localized, destructive agent. The average energy release, which, as will soon be seen, is confined within a region of 7 Å radius (on the average), is 110 electron volts or 2,500,000 calories/mole. It is likely that all ionization energy releases exceed 25 ev. This will destroy any sensitive function in its vicinity. So, by bombardment in this way, the loss of certain kinds of viral behavior can be studied. There are many of these, and for each there can be found a volume or an area related to the bombardment. Thus loss of infectivity, serological affinity, hemagglutination ability, bacterial killing power, and ability to adsorb, can each be measured. In the very limited number of

cases where several properties of a virus have been studied, the behaviors have been different. This is of great importance because each function probably occupies a different part of the virus, and the radiation action is demonstrating the fact.

To repeat in a rather different way, the use of radiation is aimed at exploiting the space relationships of high-energy releases in terms of effect on the virus. It is, accordingly, vital to know what these relationships are, and to conduct experiments so that the original relationships are retained.

Nature of Energy Loss by Fast Charged Particles

The energy loss of a fast, charged particle occurs as a result of its passage near an atom. If it causes an atom to become excited or to lose an electron (i.e., to be ionized), the energy gained by the atom is lost by the particle. This process occurs in terms of a probability only. That is to say, the fast, charged particle can approach 100 atoms in exactly the same way, and for, say, five of these, some sort of excitation can happen whereas the other 95 are *entirely unchanged*. This method of operation is that required by modern atomic theory. The probability of 0.05 is determined by some quite simple considerations, but it is always no more than a probability.

The considerations are as follows. Suppose a charged particle (e.g., a proton) is approaching an atom situated as at A in Fig. 3.1a. As the particle moves along its track, the electric field at A takes values something like the indications in Fig. 3.1b. Now while this field is present, the atom, or molecule, is in a highly strained condition, and as a result of this strain may change its configuration to one of the possible excited states, or it may ionize, or, if in a molecule, dissociate. If the flying particle is fast, the field, as a function of *time*, rises and falls rapidly. If it is slow, the rise and fall is relatively slow. This is indicated in Fig. 3.1c. This time plot shows two very important features of radiation action. First, the slow particle produces the strain in the atom or molecule for a longer time and so increases the chance of response by excitation or ionization. Second, the field which rises rapidly and falls rapidly has an equivalent fre-

quency spectrum with many high-frequency components, whereas the other has mainly lower frequency components. Since we have the familiar equivalence $E = h\nu$, where E is energy, h is Planck's constant, and ν is frequency, the very-high-energy transitions are less likely for a slow particle than for a fast particle.

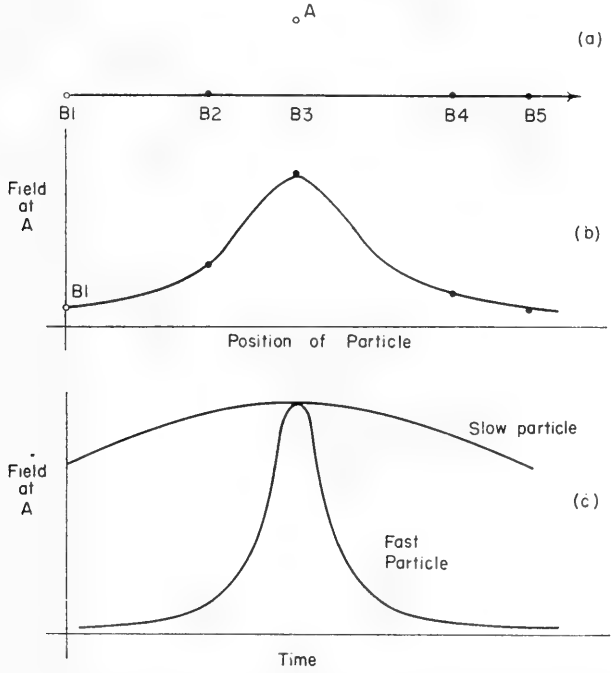


FIG. 3.1. (a) represents the flight of a fast particle past an atom at A. (b) shows schematically the electric field at A as the particle passes. (c) shows the same referred to time, allowing for the velocity of the particle.

Notice that, because all that is important is the *field*, the mass of the flying particle is unimportant.

This whole process has been subjected to rigorous theoretical analysis, for the case where A is a hydrogen atom, by Bethe (1930) and Bloch (1931). A simple and clear account of the theory is given by Fermi in his book *Nuclear Physics* (1949). The extension to more complex atoms can be made by a mixture

of experiment and theory. Two basic relations are derived. The first concerns the number, dn, of ionizing processes (primary ionizations) of energy between W_1 and W_2, and is

$$dn = \frac{2\pi z^2 e^4}{mv^2} NZ \left\{ \frac{1}{W_1} - \frac{1}{W_2} \right\} dx \tag{3.1}$$

where z is the number of elementary charges on the flying particle, e is the electronic charge, N is the number of atoms traversed per unit length, Z is the number of electrons per atom, dx is the distance traveled, m is the mass of the *electron*, and v is the velocity of the flying particle.

If we consider energy-loss values between W and $W + dW$, then the above formula becomes

$$d^2n = \frac{2\pi z^2 e^4}{mv^2} NZ \frac{dW}{W^2} dx \tag{3.2}$$

where, now, d^2n is the number of primary ionizations of energy between W and $W + dW$ in the length of path dx.

The rate of loss of energy is also important. In principle, it is given by the integral of Eq. 3.2 over all possible values of W. This is complicated by the fact that a somewhat different relation holds for *excitations*, which have discrete energies, and by the fact that both an upper and a lower limit to the possible values of W exist. The upper limit is set by the fact that energy transfers in excess of the conservation of momentum may not take place, and the lower limit by the fact that the first excited state of the atom must be reached. When these are duly processed, the result obtained (for heavy particles, not electrons) is, to a first approximation,

$$-\frac{dE}{dx} = \frac{4\pi e^4 NZ}{mv^2} \ln \left\{ \frac{2mv^2}{I} \right\} \tag{3.3}$$

where $-dE/dx$ is the rate of energy loss with distance, and I is an equivalent excitation potential which must be found by experiment.

Equation 3.3 has been checked very accurately in nuclear physics and cosmic-ray work. Its use, within the proper approximation limits, is therefore justified.

For electrons there is the added feature of the possibility of exchange between the flying electron and an orbital electron. This modifies the result to

$$-\frac{dE}{dx} = \frac{2\pi e^4 NZ}{mv^2}\left\{\ln\frac{mv^2E'}{2I^2(1-\beta^2)} - \ln(1-\beta^2) + 1 - \beta^2\right\} \quad (3.4)$$

where E' is the kinetic energy of the electron, and β is the ratio of electron velocity to that of light.

One important point arises regarding loss of energy by compounds. Chemical binding runs around 5 ev per bond, whereas ionization processes are around 100 ev. It is therefore reasonable that chemical binding should not affect particle energy loss, to a good approximation. Preiss has verified this for C_2H_2, C_2H_4, and CH_4, where the loss of energy is measured to be essentially that of an atomic mixture in the right chemical proportions. Some useful values of I are given in Table 3.1.

TABLE 3.1
VALUES OF THE EFFECTIVE EXCITATION POTENTIAL FOR ELEMENTS

Element	I (ev)	Reference
H	16	Siri (1949)
C	64	Preiss and Pollard (unpublished)
N	81	Siri (1949)
O	99	Siri (1949)
P	173	Siri (1949)

Returning to viruses, the energy lost in traversing the diameter of southern bean mosaic virus by various classes of particle is given in Table 3.2.

It can be seen from the table that a 0.5-Mev deuteron, which can readily traverse a virus particle, releases a very large amount of energy per virus. Loss of function after receiving this much energy is not surprising. On the other hand, for a 10-Mev pro-

TABLE 3.2
RATE OF ENERGY LOSS BY VARIOUS KINDS OF PARTICLE

Particle	Energy loss in ev per 100 Å in average protein	Loss in SBMV
0.5-Mev deuteron	839	2,517
1.0-Mev deuteron	597	1,791
3.0-Mev deuteron	279	837
10.0-Mev deuteron	97	291
10.0-Mev proton	64	193
10.0-Mev alpha particle	746	2,240
2.0-Mev electron	2.75	8.25

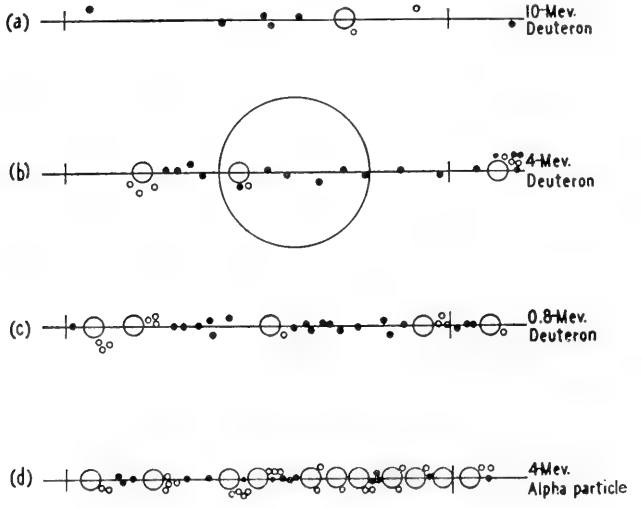
ton, the loss of energy is less by a factor of 10, and for a 2-Mev electron it is quite small.

Space Distribution of Primary Ionizations

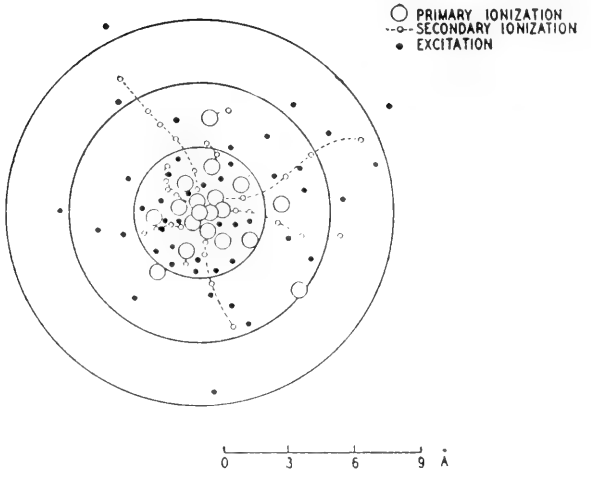
The actual energy loss is in no sense a continuous process but occurs in discrete events, primary ionizations and primary excitations. The number of the former along an electron track can be measured in a Wilson cloud chamber, and in this way the average energy release per primary ionization can be estimated. This can be done, and the process is described by the author (Pollard, 1953). The figure found is 110 ev, on the average, for a primary ionization. Associated with each primary ionization are about three primary excitations with an average energy of 10 ev each. Each primary ionization has secondary electron tracks associated with it, but the ionization produced by these, averaging two ion pairs, is mostly within 7 Å of the primary event.

The picture to carry in mind is represented in Fig. 3.2. The kind of energy loss for four representative particles along the length of the track is shown in Fig. 3.2a. The ionization and excitation events are confined to about 5 Å distance from the track for a deuteron, and about 25 Å for an electron. In Fig. 3.2b, the appearance looking down the track is shown. The virus traversed is supposed to have a thickness of 500 Å.

Exploitation of these space relationships can be made in three ways. (*a*). Heavy particles, which are unavoidably randomly



(a)



(b)

FIG. 3.2. Events along, (a), and across, (b), the track of fast particles. The large circles are primary ionizations, the small circles secondary, and the black dots are excitations. The very large circle in (a) is about the size of a protein molecule, as the vertical dashes are 100 Å apart.

distributed in area, are fired at the virus, and it is supposed that along the track there is dense enough ionization to produce a detectable effect. For this purpose, slow deuterons or alpha particles are suitable. The resulting measurement yields a "cross section," or equivalent area.

(*b*). Faster heavy particles, which ionize more sparsely, are used for bombardment. These produce primary ionizations spaced somewhat like the dimensions of the element of the virus under study. By varying the particle speed, and hence the ion spacing, the effective depth of the radiation-sensitive region can be studied.

(*c*). Fast-electron bombardment is used. This essentially gives ionizations which are random in volume because the ionization is sparse, is laterally spread, and the electrons scatter readily. This kind of bombardment measures the volume of the sensitive element.

By this triple attack, it is possible to get an estimate of the size and shape of a single, sensitive element, or the size and number of a multiple element. The method is relatively new in this kind of application but has yielded quite valid information in studies of enzymes, hormones, and antibiotics. These studies are described in the review article quoted (Pollard, 1953), and, to summarize the findings briefly, it can be said that the arrival of a primary ionization in a dry enzyme or hormone, in 15 separate measured cases, results either in the removal of its biological function, or in the removal of a definite fraction corresponding to a definite unit of substructure (as for catalase). The premise that a primary ionization can be used to "feel out" the sensitive shape is therefore justified, as far as a preliminary study goes, at all events.

In using the method, it must be borne in mind that in the preceding description no explanation of the action of ionizing radiation has been given. All that has been done is to suppose it is drastic and disruptive. In actual fact, evidence is accumulating to the effect that only some of the observed consequences of ionizing-radiation action in the dry state are due to such high-energy disruption. A part is more gentle and possibly more

widespread. This shows as a part which is temperature sensitive and also possibly depends on the previous treatment of the virus. As understanding of this dual action proceeds, radiation studies will become more powerful.

Experimental Methods and Some Results

It has been pointed out that the space relationships of ionization must be retained in the bombardment technique. To be reasonably sure of this, the virus must be bombarded dry and in vacuum. It will be seen later that this is not absolutely necessary, but this conclusion can not be drawn until dry bombardment has been carried out. Dry bombardment of TMV by X-rays was first employed by Gowen (1940) and by Lea, Smith, Holmes, and Markham (1944), who were clearly aiming at exploiting the physical action of radiation just described. X-radiation of sufficient intensity requires somewhat specialized equipment. Much shorter exposure times can be used if electrons, deuterons, or alpha particles are used. Lea made some use of alpha-particle bombardment, but, until recently, radioactive sources of sufficient purity, homogeneity, and intensity have been hard to come by.

The modification of a cyclotron for deuteron bombardment is shown in Fig. 3.3 in schematic fashion. The deuteron beam is defined by an insulated, positively charged diaphragm, and enters a bombardment chamber without striking any metal except a brass shutter or the sample holder. The beam entering the chamber is slowly diverging and, because the bombardment chamber is 8 ft from the cyclotron vacuum chamber, is uniform in cross section over a diameter of about $\frac{5}{8}$ in. The beam is measured by a calibrated galvanometer which reads current collected by the whole bombardment chamber, which is insulated. The samples are placed on glass coverslips attached by a spot of grease to a brass disk. These are shown in the lower part of the figure. These are successively rotated into place with the cyclotron off, the beam is turned up from the control room, and the shutter is electrically operated from there for the appropriate time.

Deuterons of different energy are obtained by putting foils over the samples. The whole unit is evacuated to the cyclotron vacuum of about 10^{-5} mm mercury.

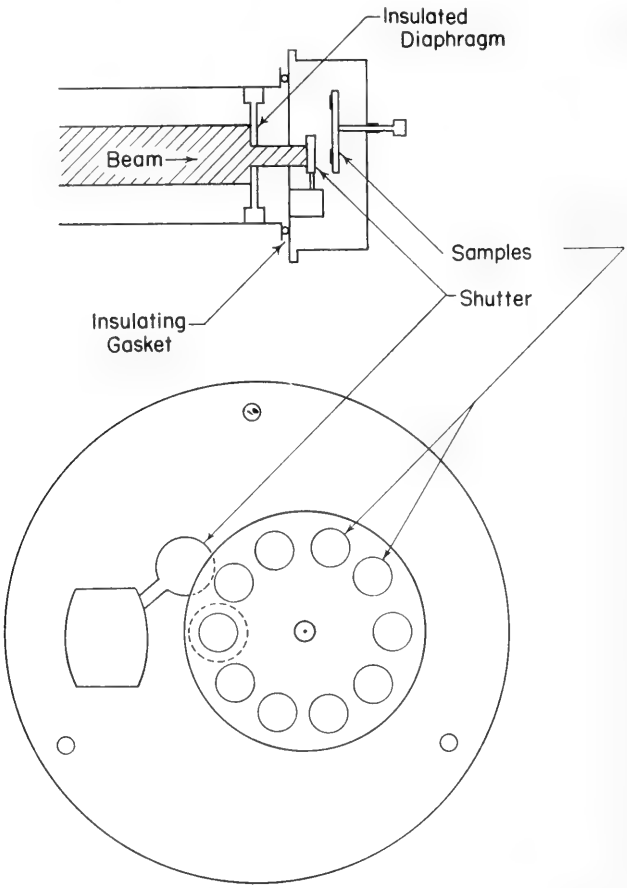


FIG. 3.3. Use of a deuteron beam from a cyclotron to bombard viruses. The upper drawing shows the side view, and the lower is an enlarged view of the end with the sample holder and shutter indicated schematically.

Results of deuteron bombardment of T-1 bacteriophage are shown in Fig. 3.4. The data are due to Pollard and Forro (1951). It can be seen that a semilogarithmic relation between the percent of infectivity surviving and number of deuterons per square centimeter employed in bombardment holds. This is the charac-

teristic relation for random bombardment which is expected to produce the result (see, for example, Pollard, 1953)

$$\ln \frac{n}{n_0} = -SD \tag{3.5}$$

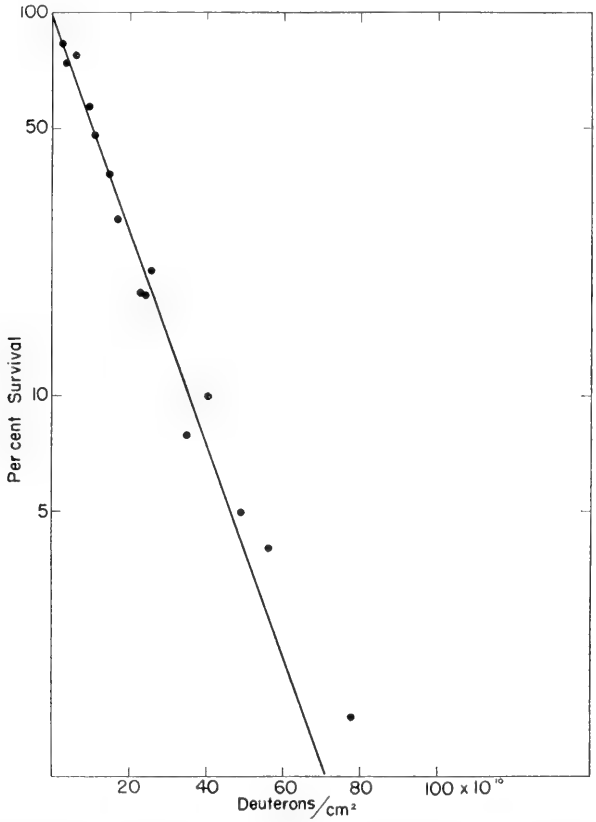


FIG. 3.4. Percent survival of T-1 phage after various amounts of deuteron bombardment. The relation $\ln (n/n_0) = -SD$ is seen to be obeyed (Pollard and Forro, 1951).

where n_0 is the initial measure of infectivity, n is the amount left after a "dose" D of deuterons per unit area, and S is the effective cross section. S is determined from the above relation, and the problem of interpretation is to take this theoretical number and show its significance in virus structure. It will be

seen later that this is simple for some viruses. It is quite hard for T-1.

In Fig. 3.5 are shown some unpublished results of Fluke, who subjected T-1 phage to electron bombardment, using 2-Mev electrons from a Van de Graaff accelerator. Again, a semilogarithmic relation is found when percent survival is plotted

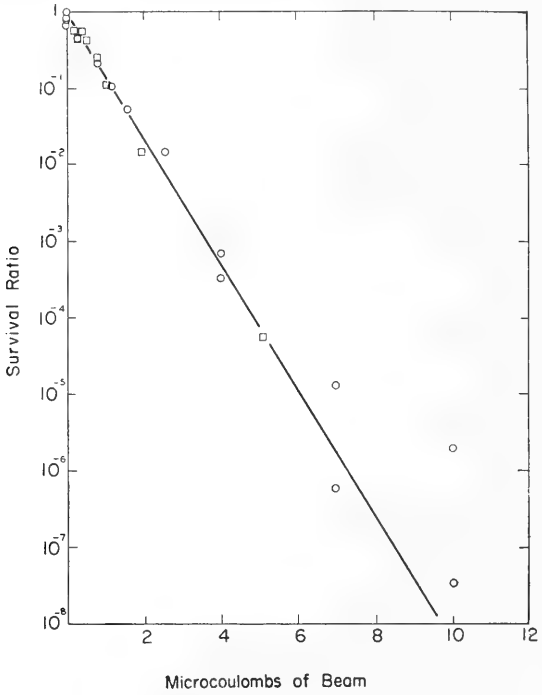


FIG. 3.5. Electron bombardment of T-1 phage. The survival ratio fits the relation $\ln (n/n_0) = -VI$. Data due to Fluke.

against primary ionizations per cubic centimeter. This relation corresponds to a bombardment which is random in volume, and so fits the equivalent relation

$$\ln \frac{n}{n_0} = -VI \tag{3.6}$$

where V, the inactivation volume, now takes the place of S, the cross section. I is the number of primary ionizations per

cubic centimeter. Again, the interpretation of V in terms of virus structure has to be made.

The third type of experiment is illustrated by Fig. 3.6 where the effective cross section, S, taken from deuteron bombardment experiments, is plotted against the energy loss on the part of the deuteron for four viruses: TMV, SBMV, T-1 *coli*, and M-5 *megaterium* phage. The deuteron energy loss is varied by

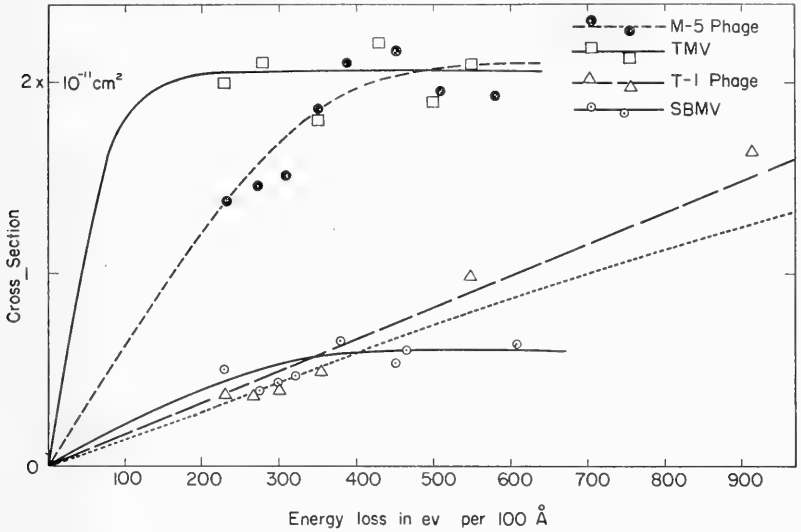


FIG. 3.6. Inactivation cross sections of TMV, M-5, SBMV, and T-1 for bombardment by deuterons of various energies and, hence, rates of energy loss. The cross section approaches a maximum for TMV, M-5, and SBMV, but not for T-1.

interposing foils in the path of the beam, thus reducing the velocity of the particle and increasing the rate of energy loss. Three types of curve are seen which illustrate three classes of virus and probably express three different internal morphologies. The first, for TMV (a purified preparation assayed by lesion count on *Nicotiana glutinosa*), shows no change in S as a function of ion density, or rate of energy loss. Within reason, S corresponds to the whole area (actually 80%) of the infectious unit of TMV. The second, for southern bean mosaic virus, shows an increasing cross section which flattens at high ion density

to give an area which is about that of the whole dried virus. The value of S for more sparse ionization is definitely less. The third case, a large bacterial virus, M-5 *megaterium* phage, shows a definite flattening in cross section area, and so is similar to SBMV, but also has a lower cross section for fast deuterons. The total cross section at high deuteron energy loss is probably less than that of the whole virus, but since it is a definite value over a finite range of energy loss, there seems to be reason to believe that a definite part of the virus is radiation sensitive. The fact that not all of a large virus need be radiation sensitive was pointed out by Lea (1947). The fourth case, of T-1, shows a steady rise in effective cross section, which may perhaps be showing some flattening if correction is made for the fact that alpha-particle bombardment, which was used for the extreme point, brings with it a larger number of energetic, secondary electrons (delta rays), and these may give an excessively high apparent efficiency to an alpha particle. This correction is indicated by the dotted line.

Analysis of Bombardment Results

It has been stressed that these findings are to be expressed in terms of the space relationships of energy loss by the bombarding particle. These are most simply (but not fully) described in terms of the occurrence of average primary ionizations at an energy cost of 100 ev per event. Using this description, the events in the three cases are shown schematically in Fig. 3.7. In this representation, the two extremes of low- and high-energy loss are shown in terms of the distribution of primary ionizations (black dots) along the particle track. It is clear that for TMV one primary ionization removes the infectivity. For SBMV, more than this is required, but probably between one and three represents the number which will destroy infective function. On the other hand, for T-1 there is a steady increase in effectiveness as the deuteron specific ionization becomes greater.

It is clear that all viruses do *not* behave in the same way as regards primary ionization. The only simple case that can be easily treated is TMV. The assumption can be made that one

primary ionization anywhere in some sensitive volume can inactivate the virus. Then the results of electron bombardment and deuteron bombardment can be combined to deduce the shape of the virus. This has been done by Pollard and Dimond (1953) as follows. The experimental deuteron cross section is 19×10^{-12}

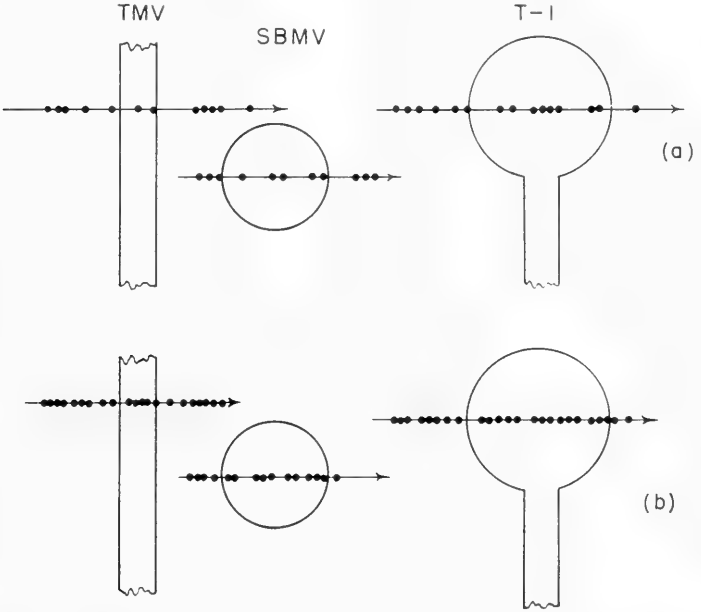


FIG. 3.7. A schematic representation of the passage of (a) a deuteron losing 200 ev per 100 Å, and (b) 600 ev per 100 Å, through three viruses. In all cases, more than one primary ionization occurs in the virus. This inactivates purified TMV, but more energy is needed for SBMV, and this is supplied in case (b). T-1 can survive even this. T-1 thus presumably has a more complex morphology.

cm^2. Assuming that the target is randomly oriented, the true cross section is $4/\pi$ times this, or roughly 24×10^{-12} cm^2. Electron bombardment gave an inactivation volume of 3.1×10^{-17} cm^3. Suppose the virus is a cylinder of radius r and length l. Then

$$2rl = 27 \times 10^{-12} \text{ cm}^2$$
$$\pi r^2 l = 3.7 \times 10^{-17} \text{ cm}^3$$

which lead to $r = 98$ Å, and $l = 1{,}200$ Å. The radiation data therefore fit a long, thin virus, rather fatter and shorter than that fitted by the electron microscope data. The measurements are not accurate enough to do more than claim a fair check and to conclude that not quite all (about 80%) of the virus is highly sensitive.

Unfortunately, TMV is the only virus so far studied for which a consistent analysis can be made. To show the kind of trouble encountered, the figures for SBMV are here analyzed. The deuteron cross section is 6.2×10^{-12} cm^2. The inactivation volume from electron data is 3.4×10^{-18} cm^3. Now we have already stressed that SBMV is spherical (though it may not be so in the dried state). So if we assume a radius r, we have

$$\pi r^2 = 6.2 \times 10^{-12} \text{ cm}^2; \qquad r = 141 \text{ Å}$$
$$\tfrac{4}{3}\pi r^3 = 3.4 \times 10^{-18} \text{ cm}^3; \qquad r = 93 \text{ Å}$$

Although these are not way out of line, from a crude point of view, the "electron" radius is too small. Also, if the critical volume is as thick as even 186 Å (twice the "electron" radius), then deuterons of low ion density should certainly produce one primary ionization in it. Yet if they do, they seemingly don't always inactivate, because S is not the full value for fast deuterons.

Notice that one figure seems to be very reasonable. The radius for maximum deuteron action is a little less than that of the virus in solution, and corresponds rather closely to the radius of the virus less hydration as observed in the electron microscope.

To weasel out of the dilemma, it can be supposed that as the virus dries it flattens. The actual sensitive volume is then less than that for the spherical virus. Assuming it to be a flat cylinder, of height h and radius 141 Å, the value of h turns out to be 76 Å. This effective thickness fits the variable-energy deuteron data of Fig. 3.6 quite well, for in such a thickness, the chance of a deuteron passing through without producing a primary ionization is 22%, so that the observed smaller cross section is quite plausible. The loss in volume from the X-ray scattering volume is 10.4×10^{-18} cm^3, which is rather great to correspond to the known hydration of the virus. There is, there-

fore, something more about the action of ionizing radiation on SBMV which must be invoked to explain the data. Further studies will be needed to bring this out.

The case of M-5 phage is somewhat similar to SBMV. The deuteron cross section for very high ion density is 2.05×10^{-11} cm^2, which is not far from that of the whole virus as seen in electron micrographs. The electron inactivation volume is not in agreement with the above figure for a spherical sensitive region. The value found is 4.0×10^{-18} cm^3, and applying the same considerations as for TMV, without correction for random orientation, we find $\pi r^2 l = 4.0 \times 10^{-18}$ and $2rl = 2.05 \times 10^{-11}$, so that $r = 12.5$ Å and $l = 8{,}200$ Å. The sensitive part is, therefore, long and thin and must be coiled up in the virus. Unfortunately, this does not fit the ion-density curve, for this indicates that there is an average of one primary ionization per target thickness at an energy loss of 250 ev/100 Å, giving a target thickness of $100 \times 110/250 = 44$ Å. The diameter calculated above is 25 Å, about half of this. It is once again likely that a critical energy must be expended in M-5 phage before inactivation can occur.

There seems, however, to be no doubt that the sensitive volume is long and thin, perhaps of the order of $50 \times 3{,}000$ Å.

Turning to the fourth virus, T-1 phage, there is a clear necessity for some added feature beyond the sensitivity of a certain part of the virus to one primary ionization. At all events, this part can not be concentrated in one volume. In the first place, no definite maximum area can be assigned. In Fig. 3.6 an attempt to correct the cross section for fast secondaries (delta rays) has been made and is shown as the dotted line. Making a guess at the maximum value we get 15×10^{-12} cm^2. The inactivation volume found by Slater (1951) for electron bombardment is 2.4×10^{-18} cm^3. Treating the sensitive volume as spherical, the radius from the first result is 218 Å (rather less than the electron-micrograph radius of the head, which is 250 Å), but from the second figure it is 83 Å, which doesn't agree at all. The two figures can be reconciled by assuming a cylindrical sensitive volume, which then needs to have a radius of 10 Å and a length of 7,400 Å. This fits nothing that is known about T-1

phage. It is possible that it corresponds to a coiled-up sensitive region of nucleoprotein.

These illustrations have been chosen for a purpose. They show that the broad assumption that the whole virus is sensitive to one average primary ionization cannot be maintained and so that no simple treatment in those terms can hold. They show that certain types of radiation study seem capable of yielding results which can be interpreted now—notably, high specific ionization bombardment, which yields a cross section which is an approximation to the area of the dry, unhydrated virus particle. They show the urgent need for a better understanding of the biological action of primary ionization. This somewhat gloomy note is in reality very misleading. Radiation, in all probability, does destroy the local region where it arrives. Whether this fact will interfere with the property of the virus being studied depends on the way the virus uses its various parts. The fact that apparently *all* the parts of the virus are not in all cases absolutely necessary for multiplication is, in the end, going to make it possible, by radiation techniques, to find out something about both morphology and function. We hope to show, later in the chapter, how this can be approached.

The Action of X-Rays

Because the interest in this book is in viruses rather than in radiation action we have treated one important class of experiment at some length. In so doing we have ignored some of the more usual techniques of ionizing radiation, and have not even mentioned the most used type of radiation—X-rays—at all. In order to consider a further range of important work, some more description of the action of X-rays is necessary. We can begin by a brief statement of the utility of X-rays and how they are related to the first part of the chapter.

X-rays are high-energy photons and so are to be classed with gamma radiation. They act by reason of their electromagnetic field and do so in two major ways, by photoelectric absorption and by Compton recoil. In the former, the photon dies and the whole energy is transmitted to one atom as excitation energy,

and to an electron which escapes with high kinetic energy. The atomic excitation energy for biological atoms (H, C, N, O, P, and S) is usually around 400 ev, whereas the escaping electron carries the rest of the energy, which is not often less than 30,000 ev. So the overwhelming majority of the effect is in the escaping electron, a fast, charged particle, behaving exactly as already described. Specific studies by Guild (1952) show that characteristic absorption in phosphorus is not favored in biological action, at least to a first approximation. So as far as photoelectric absorption is concerned, X-rays merely act to put fast electrons into the specimen. Their big advantage is that the photons penetrate, so that the electrons are really released in the middle of the system.

Compton recoil is slightly more complex. The photon does not die, but suffers a change in wavelength. A fast electron is nevertheless produced here also. The longer-wavelength photon speedily becomes absorbed by nearby atoms, again with the ejection of an electron. These scattered photons can have low energy, and so the photoelectrons have low energy. This rather complicates X-ray action. Photoelectric absorption is predominant in biological material below about 50 kv, and Compton recoil is predominant above 200 kv. In either event, the major physical action of X-rays is the production of fast electrons.

The energy of these fast electrons does not exceed the equivalent energy of the photons, which is roughly that of the X-ray tube. Actually, a wide distribution of energies is present in X-ray bombardment. This is not a serious complication since, as has already been pointed out, fast, charged particles lose energy by reason of their field, and this depends on velocity for any one particle. Now the light electron very readily approaches the velocity of light, after which it effectively becomes heavier, as it gains energy, without changing speed and ionizes in just the same way as if it had less energy. For electrons above about 200,000 ev, the rate of energy loss is nearly constant. This important fact means that very little difference in biological effect is to be expected for high- or medium-energy X-rays. For very-low-voltage X-rays, below 25 kv, this ceases to be true since the

electrons generated by the X-rays are now slower and ionize more densely, so that their effects may not be due to single primary ionizations scattered at random but may have ionization concentrated in tracks.

X-rays are measured, in practice, in terms of a unit based on ionization in air. This is the *roentgen*. Radiation dosimetry in these terms is described in many accounts, notably one by Evans (1949). Of somewhat more use is a unit based on energy loss. The amount of radiation which will release 83 ergs per gram of material is called a *roentgen equivalent physical* (rep) and roughly matches the reading on an air dosimeter in roentgens. A conversion factor from rep to primary ionizations (pi) per cubic centimeter in protein is *1 rep is equivalent to* 6.13×10^{11} *pi/cm*^{3}. With this conversion factor, X-ray inactivation data, *taken under conditions where primary ionization is the inactivating agent*, can be used to give inactivation volumes by the use of Eq. 3.6 for random volume action.

Secondary Radiation Effects

We can now turn to some secondary radiation effects of importance. By far the most important of these is the action of radiation on water. Water is the most prevalent biological constituent, and any action upon it must therefore be treated most seriously. The first action on water is undoubtedly ionization. This is followed, in many cases, by dissociation, so that there will now be present the free radicals H and OH. These are uncharged and, therefore, are subject only to the force fields of chemical valence, which operate over a few Ångstrom units, or less. This is in contrast to ions which are rapidly brought together by direct Coulomb forces and so recombine. The free radicals are chemically active, and by combination can release about 5 ev of energy, so that they are also carriers of energy. Lea (1947) has estimated that the half-life for recombination of radicals formed by sparsely ionizing particles such as electrons is 2×10^{-7} sec, and for densely ionizing particles (alpha particles) is 10^{-9} sec. The rate of collision with solute molecules at a concentration of 1 gm/l he estimates as 10^{10} per sec if the solute molecular weight

is 20, as 10^8 per sec if it is 100,000, and as 2×10^7 per sec if it is 10^7. Thus for heavily ionizing particles, recombination takes place faster than collisions with large molecules, but for electron-produced radicals, collisions even with virus particles may occur before recombination takes place. Thus the radiation produces active agents of short half-life which can be transported through the water and which can be active chemically. This chemical action was discovered by Fricke (1927, 1934) and neglected by radiation biologists for several years. The discovery of radiation action in dilute solutions of enzymes by Dale (1940), and of the great sensitivity of sulfhydryl groups by Barron and Dickman (1949), has stirred up great interest in this process. Its role in virus inactivation is important but strongly dependent on the conditions of irradiation.

Three major factors about this class of indirect radiation action must be remembered. First, there is no reason to believe the *one* radical will inactivate a virus, particularly a virus such as T-1 which can survive more than 1,000 ev of primary ionization action. So the number of radicals necessary to inactivate must be known. Second, the increased collision rate with smaller molecules renders these very effective competitors for radicals, particularly so if they are small molecules that can react chemically with free radicals. Cysteine and glutathione are such molecules. Hence, if any excess concentration of such molecules, or even of gelatin, is present, competition will remove radicals by combination with these substances and so protect the virus. This protective action was discovered by Friedewald and Anderson (1940), Luria and Exner (1941), and Dale (1942). The third factor is that radical action takes place on the surface.

All these factors can be made use of in studying viruses. Estimates of the "radical yield" are not easy because it is first necessary to know the number of radicals per ionization. Taking this to be unity, which is reasonable for electrons, figures given by Lea (1947) lead to a figure of 4,000 radicals per inactivation of tobacco mosaic virus. It is quite likely, as McLaren (1951) has pointed out, that radical yield is dependent on virus surface area.

The second feature of protective action is seen in the work of Luria and Exner (1941). When wet T-1 phage is irradiated by X-rays, a semilogarithmic inactivation is found. If the inactivation volume is measured as a function of broth concentration, it can be seen that, as the broth concentration is diminished, the inactivation volume remains constant until about 0.5% broth is used, whereupon it rises sharply. The strong broth inactivation volume, and that found in irradiating dry virus (and dry broth), are very nearly the same. This is usually taken to mean that the whole of the indirect action is "protected" for 3% broth in solution. This is actually rather surprising since the physical state of a virus would certainly be expected to influence its sensitivity to radiation. It is quite possible that two factors are compensating to give an apparent identity of inactivation volume. Probably, dry virus has a rather larger inactivation volume than does virus in solution. Around the virus in solution there will be a thin layer of water which is probably not truly part of the solution and so does not contain the protective agents of broth. This may accumulate radiation energy and so give an inactivation volume that is larger than expected. Watson (1952) quotes experiments of Doermann which show that adding a strong protective agent (cysteine) cuts the inactivation volume. We are, therefore, led to the idea that in strong-broth radiation action is mostly ($> 50\%$) due to primary ionization, but that in dilute solution it is largely due to the indirect effect of secondary products produced in water.

Summary of Utility of Radiation Studies

We can now summarize the value of radiation work.

Infectivity. (*a*). Bombardment of dry virus at high specific ionization should give the virus cross sectional area.

(*b*). Variation of specific ionization should give evidence of the presence of internal structure.

(*c*). Bombardment of dry virus by electrons should give an inactivation volume to be correlated with the results of (*b*).

Detailed Virus Properties. (*a*). Dry bombardment at high specific ionization should give the total area involved for such factors as adsorption, killing power, and serological affinity.

(*b*). Varying the specific ionization should show the approximate depth of the units responsible for these factors.

(*c*). Dry electron bombardment should give the total volume of each factor.

(*d*). Wet bombardment should enable some idea to be found of the degree to which these factors are involved with the surface.

Results of Infectivity Studies

An excellent account of some of the earlier studies on viruses is given by Lea (1947). Among the first of these were studies by Gowen and Lucas (1939) on vaccinia and by Gowen (1940) on tobacco mosaic virus. The probable complication from the indirect effect of radiation was not properly realized at first, although Gowen's work on TMV includes dry inactivation. The best summary of data up to 1946 is undoubtedly that of Lea, who concludes from the data then available that the inactivation volume can be used as a measure of the virus size for the smaller viruses. It has been pointed out that this cannot be maintained in view of more recent work under what should be better conditions. Nevertheless, the use of ionizing radiation as a means of determining some critical volume of importance to infectivity seems to have been suggested by Gowen (1940), Wollman, Holweck, and Luria (1940), Lea (1940), and Luria and Exner (1941) at very nearly the same time.

Of particular interest among work done at this time is Lea's analysis of the available data on vaccinia. Remarkably advanced studies on this virus were made by Lea and Salaman (1942). The virus was irradiated dry, by gamma rays, X-rays, and alpha particles. The inactivation dose for vaccinia is 80,000 *r*. At this figure there is 37%, or e^{-1}, survival. Using the conversion factor of 6.13×10^{11} primary ions cm^3 for 1 rep, and assuming roentgens and rep to be equivalent, the inactivation volume is found to be 2.04×10^{-17} cm^3. The actual volume, judged from electron micrographs, is roughly 9×10^{-15} cm^3, so that the volume sensitive to one primary ionization is only one four-hundredth of the whole virus, in round numbers. The inactivation dose for alpha particles was 211,000 *r*, which can be reduced to number of particles per square centimeter by using the fact that 6.5×10^4

particles cm^2 correspond to 1 r (no use of rep here), and thus the sensitive area, S, can be found. It is 7.3×10^{-11} cm^2. The whole virus area is 5.5×10^{-10} cm^2, which is eight times larger. Lea proposes to reconcile all these figures by supposing that there is an internal genetic structure which is radiation sensitive. It consists of n units each of radius r, as a first approximation. Putting in the two relations $n(4/3)\pi r^3 = 2.04 \times 10^{-17}$ and $n\pi r^2 = 7.3 \times 10^{-11}$, we deduce 296 units of radius 21 Å. This is only a very first trial of such radiation analysis of internal structure, but it is significant that electron micrographs show clearly that there *is* a substructure; and 296 units in it is not a hopelessly implausible figure. The danger of this type of reasoning lies in the fact that the nongenetic part is still somewhat radiation sensitive, so that the alpha-particle cross section may contain a part which is not genetic. This would modify that conclusion to give fewer, bigger, units. When the various features of radiation sensitivity have been sorted out and understood, the deductions regarding internal structure will be of the utmost value.

Before passing on to consider some of the more recent work on viruses and ionizing radiation, we give a table of some of the measured inactivation cross sections and inactivation volumes,

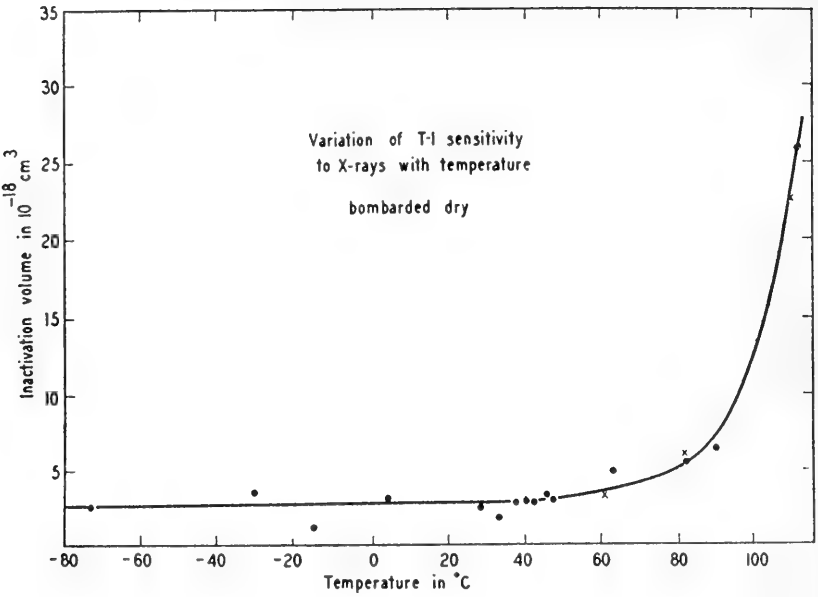
TABLE 3.3
INACTIVATION DIMENSIONS

Virus	Cross section ($Å^2$)	Volume ($Å^3$)	Virus area ($Å^2$)	Virus volume ($Å^3$)	Radiation reference
TMV	2.05×10^5	3.7×10^7	2.7×10^5	4.5×10^7	Pollard and Dimond (1953)
SBMV	6.2×10^4	3.4×10^6	6.2×10^4	11.8×10^6	Pollard and Dimond (1953)
M-5, phage	2.10×10^5	4.0×10^6	3×10^5		Friedman and Pollard (1953)
T-1 phage	1.6×10^5	2.4×10^6	3×10^5	7.5×10^7	Pollard and Forro (1951)
Influenza	3×10^5 (rough)	—	10^6	8×10^7	Woese and Pollard (1953)
Bushy stunt	5.7×10^4	3.62×10^6	5.4×10^4	7.4×10^6	Lea and Smith (1942)
Vaccinia	7.3×10^5	2.0×10^7	5×10^6	9×10^9	Lea and Smith (1942)
Tobacco necrosis	—	2.9×10^6	6.0×10^4	11.0×10^6	Lea and Smith (1942)
Newcastle disease	6.9×10^5	—	10^6	8×10^7	Woese and Pollard (1953)

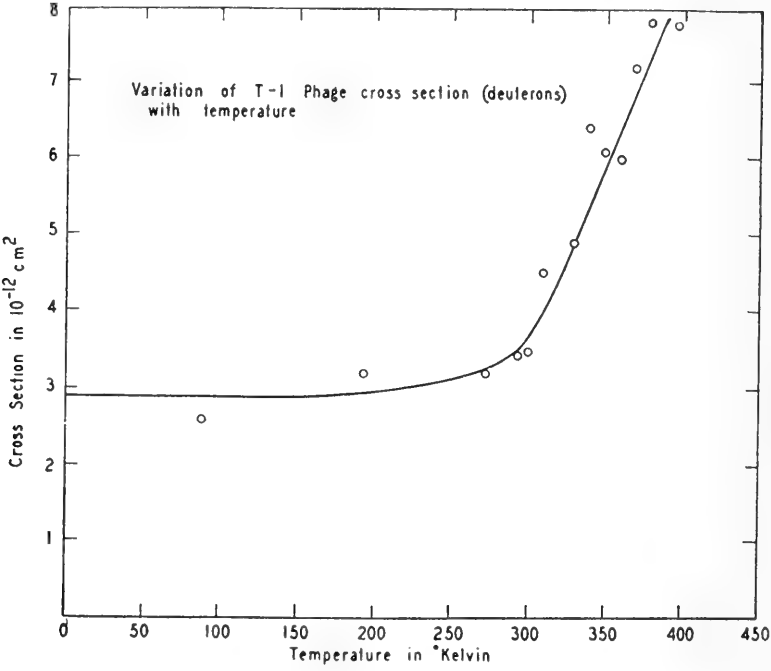
with the virus dimensions given for comparisons. It can be seen that the cross sections of the smaller viruses, as determined from heavy-particle bombardment at high rates of energy loss, are in fair agreement with the accepted area. These do not depend on the concept that one primary ionization inactivates the whole virus, but often correspond to 10 or 20 such ionizations for the effect to be produced. The volume figures, based on this assumption, do not (in general) agree with the whole virus, which can be taken to mean that there is an internal structure of different sensitivity. Radiation measurements should be able to shed some light on this. However, more must be known of radiation action before this can be determined; and to show what other features are present we consider two other experiments which bear on this, though they do not as yet clear it up.

Combined Thermal and Ionizing-Radiation Action on a Virus

Heat, or perhaps more properly, exposure to temperature, inactivates viruses. It does so by a persistent low-energy agitation of some sensitive molecular structure which after a while, aided by fluctuations, gives way to produce inactivation. The threshold energy at the sensitive point turns out to be of the order of 30,000 calories/mole or thereabouts, which is about 1.3 ev. This is far below the 110 ev of a primary ionization, and so one would think that it could confidently be asserted that changing the temperature of a virus while it is being irradiated should have no effect. This turns out to be partly true and partly not true. If the radiation is volume random, so that no more than one average primary ionization can occur in a sensitive volume at a given time, the measured inactivation volume is (for one bacterial virus—T-1) constant from $-80°$ C to 45° C. This was found by Adams and the author (1952). Above 45° C, the inactivation volume rises rather sharply. These results are shown in Fig. 3.8a. Moreover, if the radiation is densely ionizing, there is a thermal effect even as low as liquid-air temperatures, as can be seen from Fig. 3.8b. The thermal effect becomes more marked at higher temperatures, indicating that at about 45° C an



(a)



(b)

FIG. 3.8. Effect of combined primary ionization and temperature on T-1. (a) shows the effect of X-rays, and (b) deuterons. There is clearly a differential sensitivity corresponding to two types of radiation action, one intense and the other more gentle and depending on the condition of the virus.

additional effect becomes apparent. Similar results have been obtained independently by Bachofer (1953).

These results show that a virus such as T-1 cannot be regarded as homogeneous, even within that part which is radiation sensitive. The fact that there is an interplay between high-energy (ionization) and low-energy (thermal) effects indicates that either transfer of energy through the virus to a sensitive part, or damage to a larger part of the virus, is facilitated by irradiation at high temperature.

A second experiment, carried out by Adams and the author (1953), sheds some light on this question. The latent period of T-1 was studied for those virus particles which had undergone many deuteron hits but had survived. It was found that the latent period for these survivors is increased significantly, in agreement with a finding of Luria (1944) that ultraviolet light produces such action. The increase in the latent period was a function of the number of hits received, and this indicates that there is apparently a part of the virus which does not determine whether the virus will multiply but does determine the rate. The results of deuteron bombardment of T-1, with consequent action on the latent period, are shown in Fig. 3.9. The ratio of the measured latent period to that found after deuteron bombardment is plotted versus the number of deuteron hits on an assumed maximum sensitive area of 2×10^{-11} cm^2. A linear increase is observed, with a doubling of the latent period for 15 hits. Some more recent work by Fluke indicates that the burst size for radiation survivors is diminished. It is tempting to suppose that a part of the virus consists of an enzyme system which is related to the bacterial chemical content and which produces virus precursors. By damaging individual enzyme molecules, the rate of multiplication, and also the amount of precursor material available for manufacture of virus, is reduced. So we conclude that the virus contains a rather large number of enzyme molecules which are only concerned with rate of multiplication. Probably not many types of viral enzymes are involved.

The enhanced effect of temperature is thus to be thought of as taking place on this enzyme-like system. The effect of tem-

perature is, therefore, to increase the volume inactivated and not to increase the sensitivity of a highly significant target volume.

These two experiments show that the simple considerations advanced by Lea and used by him to deduce an internal structure for vaccinia cannot be applied with certainty. First, the part of radiation action which seemingly inflicts *damage*, which can

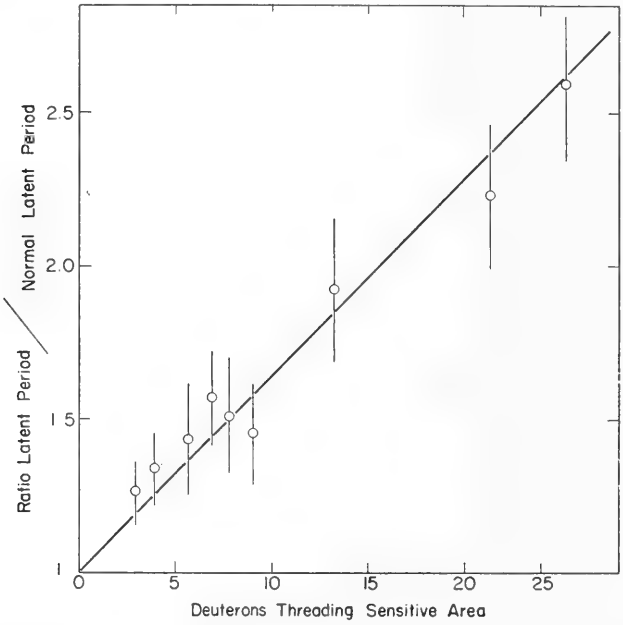


Fig. 3.9. Increase in latent period of T-1 phage due to deuteron bombardment.

accumulate to be fatal, must be ascertained and eliminated from the calculations. Then the area and volume technique can be used for analysis of internal structure.

Structural Deductions

Applying this idea to T-1 phage turns out to be rather hard because the laborious experiments to determine the nontemperature-dependent cross section at high ion density have not yet been made. The figures now available show that the measured

area and volume values require either a highly multiple genetic structure for T-1 or else a long, thin genetic structure. The best guess at present is a long cylinder of radius 26 Å and length 1,900 Å.

For the two plant viruses so far studied, the "genetic" part, which is essential for survival, seems to be very nearly the whole virus, at least the unhydrated part of the virus.

Returning to T-1, we can make some analysis of the increase in latent period. Adams and Pollard's data indicates that X-ray bombardment corresponding to 25 primary ionizations per phage particle will just double the latent period. Now if we suppose that there is some critical concentration of a virus precursor which must be reached very rapidly, we can suppose that the full burden of the manufacture of this special precursor falls on the virus itself. Perhaps it is this which converts the basterial metabolism so rapidly to virus synthesis. The 25 primary ionizations can be thought of as inactivating 25 molecules responsible for this. Since the latent period is doubled by this bombardment, the rate of precursor synthesis is halved, so that 25 molecules are left, making an initial total of 50.

Now, 15 deuterons produce the same effect. These deuterons each produce 15 primary ionizations in the virus, or a total of 225 primary ionizations. Thus the dense tracks of deuterons make an inefficient use of the primary ionizations, which indicates that each of the sensitive molecules is large—large enough to include several primary ionizations. If these hypothetical molecules are considered to be spherical, their radius must exceed 50 Å.

Actually, if we speculate that the whole virus volume is divided into 50 units, and consider these as spherical, the radius of each is 75 Å. This would make the deuteron and X-ray data roughly fit. The molecular weight of each unit would be roughly 1,000,000.

These units are possibly nucleoprotein units of some kind since the total nucleic acid in the virus far exceeds the assumed purely genetic part. This type of estimate is a mixture of speculation and reality. The fact that 25 primary ionizations

only reduce enzymatic rates by a factor of two argues that there must be many enzymes. So the existence of a multiple enzymatic structure, capable of rapidly changing bacterial metabolism, is strongly suggested by these experiments. The speculation could be reduced and the validity of description increased by better experimentation, which lies ahead.

Varied Effects of X-Radiation on Bacteriophage

In a remarkable series of experiments, Watson (1950, 1952) has studied various actions of X-radiation on T-2 bacteriophage. Watson's work is largely qualitative in that he seeks the nature of the radiation action and is only concerned with its magnitude in passing, so to speak. The studies involve many of the known properties of bacteriophage, such as adsorption, ability to kill bacteria, ability to multiply, and ability to be photoreactivated. Care was taken to distinguish between primary-ionization (direct) effects and indirect action as far as could be done. The direct effect was magnified by irradiation in broth suspension and studied first. The second paper concerns indirect action in dilute synthetic medium. The effect of X-rays on the individual phage properties was studied under the two limiting conditions for direct and indirect action.

The numerical following up of Watson's work with deuteron, electron, and ultraviolet irradiation will be of tremendous value in determining the internal structure of bacteriophage. From Watson's paper the following facts can be determined.

Adsorption. For an estimated bombardment of 1.9×10^{17} primary ionizations/cm^3, the loss of ability to adsorb was less than 5%. It can be concluded that the inactivation volume for adsorption is less than 6×10^{-20} cm^3. It is very likely that this inactivation process will turn out to be multiple hit. Valuable information about the bacterial surface should result from studying it. The molecular unit involved must have a molecular weight of less than 4,000.

Virus Activity. Measured simply in terms of virus activity the inactivation volume of T-2, T-4, and T-6 is 4.2×10^{-17} cm^3, which is one-sixth the electron-micrograph volume. This is

actually a rather larger fraction than is found for T-1 phage, and shows that some characterization of viruses in this way is possible.

Bacterial Killing Ability. The results of studying the reduction of bacterial colony counts by T-2 phage after irradiation are shown in Fig. 3.10. It can be seen that a semilogarithmic inactivation of the killing fraction takes place. The inactivation

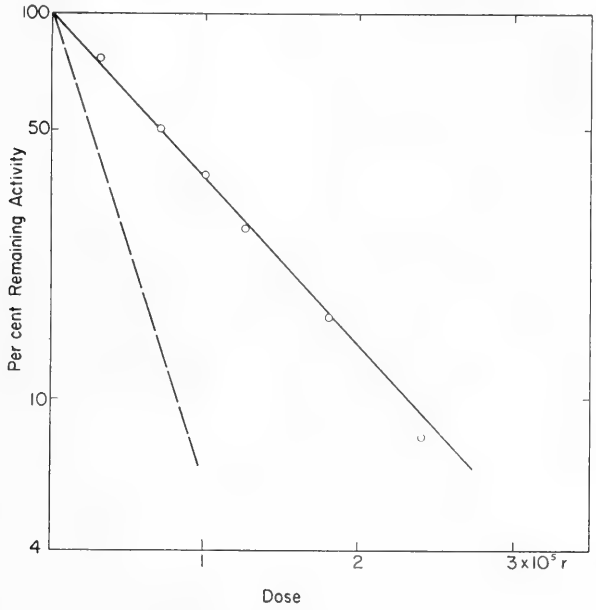


FIG. 3.10. Inactivation of bacterial killing power (solid line) and ability to form plaques (dotted line) as a result of X-ray bombardment. Data due to Watson (1950). The killing power inactivation volume is clearly less.

volume for this is one-third of that for virus activity, and so is about one-eighteenth of the whole virus.

Lysis from Without. If enough virus particles attach to a bacterium, it is lysed without being entered by the virus. This ability is not destroyed by X-irradiation even when the ability to kill has been destroyed. Presumably this property also has a small inactivation volume.

Structural Inferences from Radiation Studies

In Fig. 2.13 we showed the appearance of two plant viruses which results from studies of sedimentation, diffusion, and X-ray scattering. Since the most detailed radiation studies have been made on bacterial viruses, we show in Fig. 3.11 what can be

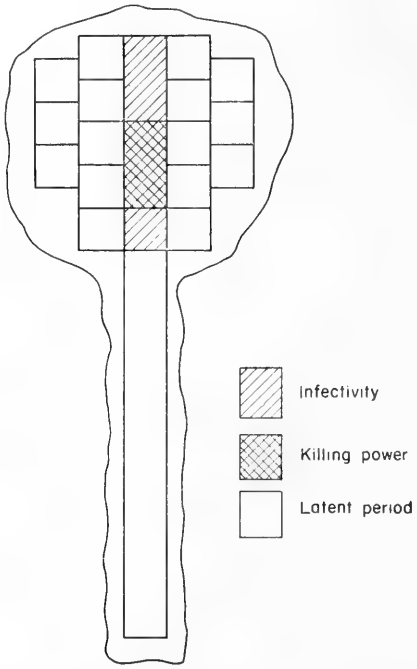


Fig. 3.11. Schematic representation of the various radiation cross sections of a T-series bacteriophage. The various factors are not put in place, but their relative areas are indicated.

inferred about bacterial virus structure from the considerations outlined in this chapter. The picture is unfortunately a composite of T-1 and T-2 bacteriophage, which is probably an unjustified synthesis of two very different viruses. This is no great detriment since careful enough studies of any one virus have yet to be made. The picture is, therefore, meant to give a general impression of two things—what the internal structure of a virus may be

like, and what factors can be determined by careful radiation study.

The part of the virus which is needed for infectivity and which can be destroyed by one primary ionization is shown as long and thin, just above the tail of the virus. That it is long and thin is probable, that it is straight is pure guessing mixed with a little laziness in drawing. The smaller killing fraction is drawn double-cross-hatched. The 50 enzyme-like units are shown, crudely to scale, and the whole is encased in some outer part which is seemingly not very radiation sensitive.

The reader should notice that this is wholly derived from electron microscopy (for the outer shape) and deductions from radiation work. The picture now has to fit other physical and chemical studies but, most importantly, it must check with all the purely biological findings. When a more complete picture, including information from ultraviolet action spectra and surface studies is drawn, an attempt to see whether the resulting picture can work will be made.

A very good analogy for the kind of information assembled in this way is a series of geographic maps of a nation in which climate, population, height, and mineral deposits are separately indicated. The sum of the maps should tell a lot about the general character of the nation, and all should mutually agree. We have here assembled an early version of one map of a virus in terms of radiation action. Others can be made and all should fit into an inferred, but true, structure.

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CHAPTER FOUR

THERMAL INACTIVATION OF VIRUSES

One of the outstanding features of living things is their instability when acted on by heat. This is not an all or nothing affair—some organisms, like bacterial spores, are stable over a wide temperature range, others are highly sensitive. Now heat is a physical agent, and the careful characterization of the action of heat on viruses should at least offer a guide in classifying them and possibly can give information about their structure.

As in the case of radiation action, we propose first to give a description of the nature of thermal action. In doing this we are aided by many studies on the thermal denaturation of proteins and the thermal inactivation of enzymes which have been made and analyzed in terms of theory. The basic theory is set out by Glasstone, Laidler, and Eyring (1941), and, more specifically for proteins, by Stearn (1949). A brief account of this theory, modified to suit the present purpose, is here presented.

Outline of the Theory of Thermal Inactivation

A virus, or an enzyme, is a large and complex molecular structure. It owes its ability to function to the fact of having inherited a certain rather precise structure from the living cell in which it originated. Heat, the thermal agitation of the atoms that form the molecular structure, is steadily and ceaselessly vibrating the atoms back and forth, and twisting and stretching chemical bonds, and so exerts a steady tendency to alter the structure. It seems likely that biologically formed molecules are not ultimately stable, by which we mean that probably some similar configurations have a lower total potential energy; but they certainly possess a local stability, by which we mean that

the functioning configuration corresponds to one minimum of potential energy, if not the lowest there is. The denaturation of such a molecule can be thought of as the changing of its configuration from the inherited form to one of perhaps greater ultimate stability but no longer of the proper form for its function.

Now a virus, as we have seen, is not a molecule but an aggregate of molecules. Each of these has a function, or plays a part in some function. The loss of biological form on the part of one of these molecules may not necessarily influence the behavior of the virus—it will depend on what feature of its activity we are studying. If, of course, there exists some function which requires the entire virus to be intact, then the loss of this function would take place if one molecule were denatured. In practice, it seems as though infectivity is a function which requires at least a great part of the virus to be intact, and probably loss of infectivity follows when one key molecule is inactivated. This will certainly vary from virus to virus.

Therefore, as a start, we consider the process of inactivating one large biological molecule and see whether the description of the process is apt for the description of inactivating viruses.

In Fig. 4.1 is a token representation of part of a protein molecule. We have taken the Pauling-Corey-Branson (1951) helical structure as a basis for the figure, but this is not essential. There are three classes of bond: The backbone bond, which binds the polypeptide chain, consists of covalent bonds of energy between 3 and 5 ev per bond. These appear in the figure as the bonds forming the helix. There are then the intrachain bonds which make the helix tight and firm, these appear as dashed lines; and the interchain bonds which hold the helices together. In the figure, these are shown as thick dashed lines. These last two types of bond are probably varied in character and include sulfur bridges, hydrogen bonds, and ionic bonds, but they are certainly weaker per bond than the covalent backbone bonds.

Such a molecule, possessing, say, eight chains bound together, although nowhere bound with great strength outside of the polypeptide chain, has a considerable stability. It is instructive to construct a model of even one chain and notice that the

breaking of one bond does not seem to destroy the configuration. The nearby breaking of several does produce a marked collapse, and it is tempting to think of this as the process involved in denaturation.

Now each bond vibrates, or can do so, and, because it is a confined system, the laws of quantum mechanics require that

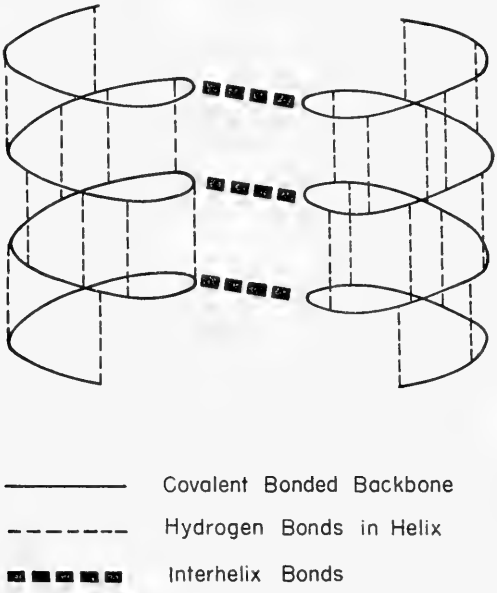

FIG. 4.1. Schematic representation of the three classes of bond in a protein. The helix has considerable binding energy per bond but the hydrogen bonds in each chain, and the interchain bonds, are weaker.

only certain restricted vibrational energy levels are possible. We definitely do not intend to go into this whole subject, but we do need to assume that there is a ladder of levels for each form of vibration, and so we will assign to the rth level of a ladder the energy E_r. If n of the possible bonds are in the energy level r, and n_0 in that of the ground state, then the most likely configuration at a temperature T degrees Kelvin is one for which

$$\frac{n}{n_0} = e^{-E_r/kT} \tag{4.1}$$

where k is Boltzmann's constant. The free energy, F, corresponding to this class of bond is then (Schrödinger, 1948, p. 13)

$$F = kT \ln \sum_r e^{-E_r/kT} \tag{4.2}$$

and by the definition of free energy in thermodynamic terms

$$F = U - TS$$

where U is the internal energy and S the entropy.

Now U, the internal energy per form of vibration, is

$$U = \frac{\sum_r E_r e^{-E_r/kT}}{\sum_r e^{-E_r/kT}} \tag{4.3}$$

which is simply the energy times the population divided by the population to give total energy. We can, therefore, use these expressions to find S, the entropy involved.

We then have explicitly: The *internal energy*, U (*or* H), *per form of vibration* is

$$U = \frac{\Sigma E_r e^{-E_r/kT}}{\Sigma e^{-E_r/kT}}$$

The total internal energy will be the sum of terms of this kind.

The *free energy per form of vibration* is

$$F = kT \ln \sum_r e^{-E_r/kT}$$

The total free energy is again the sum of terms of this kind.

The *Entropy*, S, for the case where no volume changes is

$$S = \frac{\sum_r E_r e^{-E_r/kT}}{T \sum_r e^{-E_r/kT}} + k \ln \sum_r e^{-E_r/kT} \tag{4.4}$$

Again this must be summed for each form of vibration.

Returning to the schematic drawing of part of a protein molecule shown in Fig. 4.1, the effect of thermal agitation is to cause excitation of many of the weaker bonds and, possibly to a lesser extent, of the covalent bonds. As the temperature increases, this excitation increases. The excitation is not uniform, except on a long time average, but abnormal excitations can occur. If one such causes a bond to break, there is a chance that a general irreversible modification of the molecule can follow. This modification may destroy the biological function and, if it does, the assay procedure will detect it as an inactivated molecule.

The number, dn, of such molecules inactivated will clearly depend on the time, dt, and on the number, n, of intact molecules present but, because it is not concerned with reaction with external agents in the chemical sense, it will not depend on concentration in any way. The reaction equation is then $-dn/dt = k_1 n$, where k_1 is the reaction constant. This can be integrated to give the relation $\ln (n/n_0) = -k_1 t$, where n/n_0 is the fraction of activity remaining at time t. This is usually easily measured, and so the measurement of k_1 is inherently not a difficult matter.

Now the theory of absolute reaction rates (see Stearn, 1948) states that if a monomolecular-type reaction that obeys the above relation takes place, then

$$k_1 = \frac{kT}{h} e^{-\frac{\Delta F^\ddagger}{RT}} \tag{4.5}$$

where $\Delta F^\ddagger$ is the free energy of activation for the process, R is the usual gas constant and h is Planck's constant.

This can be written in terms of the heat of activation, $\Delta H^\ddagger$, which is the change in internal energy for volume change, and the entropy, $\Delta S^\ddagger$, of activation by using the constant-volume relation

$$\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger$$

If the variation in volume warrants it, the relation

$$\Delta F^\ddagger = \Delta H^\ddagger - T\Delta S^\ddagger + P\Delta V^\ddagger$$

must be used.

Consider now the three predominant classes of bond. The covalent bond has widely spaced energy levels, so that, for thermal purposes, only two or three values of the energy need be considered. The helix bonds and the interhelix bonds have more closely spaced levels, and so more energy values need to be considered.

Eyring's theory of the activated state supposes that if F is plotted against a general reaction coordinate, which is, broadly speaking, a bond length, then there exists a second configuration separated by an intervening hump as shown in

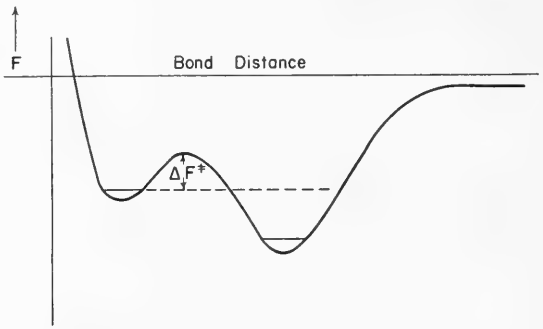

FIG. 4.2. Representation of a free-energy potential barrier which must be crossed to permit inactivation of a biological molecule.

Fig. 4.2. For a large molecule to attain the energy $\Delta F^{\ddagger}$ certainly involves many other bonds as well, so that $\Delta F^{\ddagger}$ includes changes in covalent, helix, and interhelix bonds.

A measured value of $\Delta F^{\ddagger}$, therefore, can be analyzed into $\Delta H^{\ddagger} - T\Delta S^{\ddagger}$, and corresponds to a total energy requirement of $\Delta H^{\ddagger}$ balanced by an entropy term which expresses the effect of a tendency to reach the most probable state.

If this expression, $\Delta F^{\ddagger} = \Delta H^{\ddagger} - T\Delta S^{\ddagger}$, is substituted into the Eyring formula for absolute reaction rates, it can be seen to yield

$$k_1 = \frac{kT}{h} e^{\frac{\Delta S^{\ddagger}}{R}} e^{-\frac{\Delta H^{\ddagger}}{RT}} \tag{4.6}$$

where, now, $\Delta S^{\ddagger}$ appears as a positive exponent which aids the speed of the reaction.

In the inactivation of enzymes and the denaturation of proteins, it is common to find values of $\Delta S^{\ddagger}$ of the order of 10–100 calories/mole/degree. These produce a drastic change in k_1, and it is in order to examine what processes can operate to give a high entropy of activation. Examining Eq. 4.4 for entropy, we see that the first term corresponds to the effect of changing the energy per state of vibration. This can take place if there is considerable expansion (and some recent work by the author indicates that, for proteins, this may be so), but, since the range of temperatures covered in biological inactivations is quite small, the predominant cause of an entropy increase is the second term. This means there is an increase in the number of available forms of vibration. Two major reasons for this exist: the first is the opening up of chains, which permits a whole class of pendulum-like oscillations to take place; and the second is the release of bound water which is now free to rotate in two degrees of freedom per molecule where before these possible modes were held down. So when figures are seen later regarding entropies of activation, they are to be associated with these two possibilities. It will be seen, as is reasonable, that in the dry state activation entropies are quite small, so that probably a very large part of the entropy of activation is concerned with hydration.

Thermal Inactivation of Viruses

Many measurements of the thermal inactivation of viruses have been made since this is a primary piece of knowledge in the handling of pathogens. Not very many are suitable for measuring rate constants and so are not suitable for determining the activation—heat change and entropy change just discussed. The usual procedure is to describe the 10-min thermal inactivation point, which is obviously a very practical piece of information but is, alone, not too informative from our point of view.

The fact that the thermal inactivation of a bacterial virus follows first-order kinetics was shown by Krueger (1931), who studied a *staphylococcus* phage. Careful studies on four plant viruses were made by Price (1940). Some of his results are

shown in Fig. 4.3. The natural logarithm of the virus concentration, as determined by counting local lesions on the appropriate plants, is plotted against time, in minutes, for the four viruses tobacco ringspot, tobacco mosaic, tobacco necrosis, and alfalfa

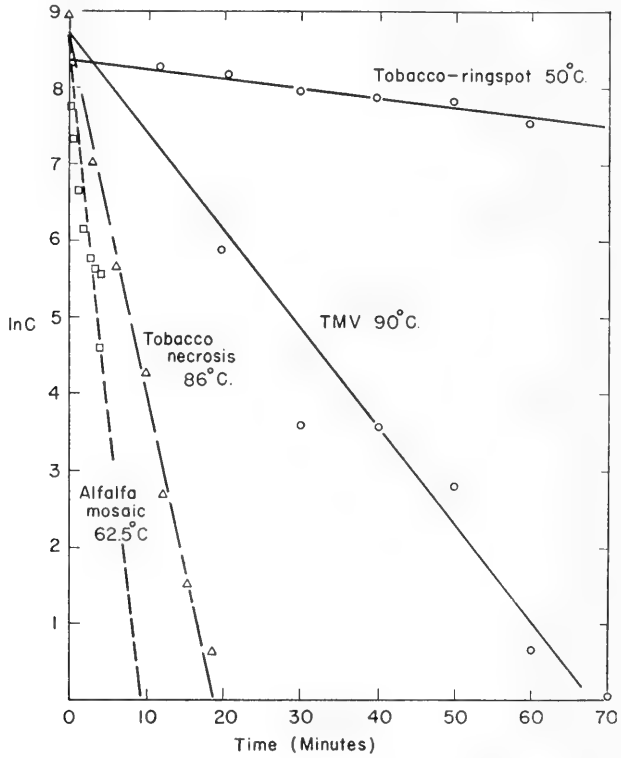

FIG. 4.3. Inactivation of four plant viruses as measured by Price (1940). The logarithm of the concentration is plotted versus time, and first-order kinetics are obeyed.

mosaic at the temperatures indicated. In the case of tobacco mosaic virus, Thornberry, Valleau, and Johnson (1938) have studied the thermal inactivation in the dried leaf of the host plant, white burley tobacco, over a very wide temperature range.

The data on the dried virus just mentioned can be analyzed according to the Eyring relation. The resulting values found

are $\Delta H^{\ddagger} = 25{,}300$ calories/mole and $\Delta S^{\ddagger} = -7.4$ calories mole/degree. Some unpublished experiments by the author and Dimond on purified, dry TMV give $\Delta H^{\ddagger} = 27{,}000$ and $\Delta S^{\ddagger} = 0$. The dry virus is, therefore, characterized by a low or zero entropy of activation and a moderate value of $\Delta H^{\ddagger}$. The low entropy is characteristic of dry substances (Pollard, 1951).

The wet inactivation yields two sets of figures. Below about 85° C, the reaction constant varies relatively slowly with temperature, yielding $\Delta H^{\ddagger} = 40{,}000$ and $\Delta S^{\ddagger} = 18$ in some experiments made by the author and Dimond and which substantially agree with the data obtained by Price. Above about 85° C, the temperature dependence rises markedly. Price's figures for the undiluted virus correspond to a $\Delta H^{\ddagger}$ of 195,000 carlories, mole and a $\Delta S^{\ddagger}$ of 410, roughly. The very much larger value of $\Delta S^{\ddagger}$ above 85° C speaks for a completely different process taking place. Lauffer and Price (1941) studied the denaturation of TMV with thermal action. TMV behaves in a rather interesting manner at high temperatures. The small fraction of nucleic acid is removed from the protein binding, the protein becomes denatured, and goes out of solution. The rate processes for this were carefully measured by Lauffer and Price (1941) with the result that, at *p*H 6.8, the values of 153,000 for $\Delta H^{\ddagger}$ and 370 for $\Delta S^{\ddagger}$ were obtained.

It is thus clear that infectivity and protein denaturation do not necessarily go together. The latter is characterized by very large entropies of activation, and the former by lower values. If the temperature is high enough, the thermal action on the protein part will stop the virus from functioning faster than will the other process, whatever it may be, which was causing the infectivity loss. It will be seen later that the loss of serological affinity follows a high-entropy type of kinetics and so is presumably related to protein denaturation.

The two processes are plotted in Fig. 4.4, in which the rate constants for TMV inactivation and denaturation are plotted versus temperature. The two separate processes of denaturation and infectivity loss are seen to combine in the one curve as measured by Price. It is tempting to suppose that the slow

process of infectivity loss is due to the inactivation of nucleic acid. It is regrettable that so little data on the thermal inactivation of nucleic acid measured in some biologically functional way has been assembled. Some very preliminary work by Fluke and Drew on the transforming factor for pneumococci indicates a rather moderate entropy change. It may well be

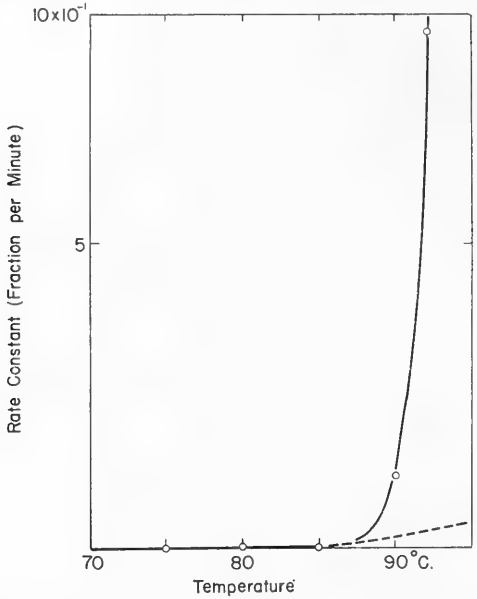

FIG. 4.4. The rate constant for inactivation (dotted line) and denaturation (sharply rising line) of TMV derived from Price's (1940) data. The slower inactivation process is superseded by the faster denaturation process at higher temperatures.

that this depends on the lightness of the combination between nucleic acid and protein.

Similar results were found for tobacco ringspot virus but not for alfalfa mosaic or tobacco necrosis virus. Cherry and Watson (1949) observed a similar effect for a *S. lactis* phage, which showed a very rapid rate of inactivation at 65° C. The values deduced from their work are given in Table 4.1.

A very careful study of the thermal inactivation of T-5 *E. coli* bacteriophage has been made by Adams (1949). He observed

TABLE 4.1
THERMAL CONSTANTS FOR THE INACTIVATION OF SOME VIRUSES

Virus	Condition	$\Delta H^{\ddagger}$ (calories/ mole)	$\Delta S^{\ddagger}$ (calories/ mole/°C)	Reference
E. Coli				
T-1 phage	dry	27,500	0	Pollard and Reaume (1951)
	wet (broth)	95,000	207	Pollard and Reaume (1951)
T-2 phage	dry	18,000	−12	Pollard and Reaume (1951)
	wet (broth)	71,700	139	Pollard and Reaume (1951)
T-3 phage	dry	19,100	−9	Pollard and Reaume (1951)
	wet (broth)	105,000	246	Pollard and Reaume (1951)
T-4 phage	wet (broth)	120,000		Adams (1949)
T-5		82,000	165	Adams (1949)
T-7	dry	12,700	−29	Pollard and Reaume (1951)
	wet (broth)	60,700	114	Pollard and Reaume (1951)
B. Megaterium phage				
M-1	wet (broth)	76,000	165	Friedman (1953)
M-2	wet (broth)	82,000	183	Friedman (1953)
M-3	wet (broth)	87,000	195	Friedman (1953)
M-4	wet (broth)	136,000	347	Friedman (1953)
M-5	wet (broth)	112,000	254	Friedman (1953)
TMV	dry	27,000	0	Pollard and Dimond (1953)
	wet (pH6.8)	40,000	18	Pollard and Dimond (1953)
	denaturing process	195,000	410	Price (1940)
SBMV	dry	17,000	−16	Pollard and Dimond (1953)
	wet	25,000	−5	
S. Lactis phage	wet 30–45° C	11,000	—	Cherry and Watson (1949)
	55–65° C	76,000	—	
Tobacco ringspot	wet 45–56° C	79,000	—	Price (1940)
	56–65° C	27,600	—	
Tobacco necrosis	wet 70–95° C	37,300	—	Price (1940)
Alfalfa mosaic	wet 50–62° C	75,000	—	Price (1940)
Influenza	wet (pH 7)	34,000	39	Lauffer, Carnelly, and MacDonald (1948)

inactivation according to the first-order kinetics already described, and his measurements of the reaction constants for virus in broth lead to the values 81,000 for $\Delta H^{\ddagger}$ and 165 for $\Delta S^{\ddagger}$. The important feature of Adam's studies, however, lies in the effect of varying the concentration of monovalent and divalent ions on the over-all process of thermal inactivation. It was found that if the phage were maintained in phosphate buffer (0.001*M*), 0.15*M* NaCl, and a little salt-free gelatin, the activity at 37° C was lost in a matter of an hour or two. Adding small concentrations of divalent ions radically changed the rate of inactivation. In following this observation further, Adams measured the reaction constants in 0.1*N* NaCl and 0.1 sodium citrate at various concentrations in the absence of divalent ions and then in the presence of various concentrations of divalent ions. The kind of effect produced is illustrated in Fig. 4.5. The velocity constant in fraction per second at 50° C is plotted against the concentration of sodium or magnesium ions. The remarkable change in value is clearly shown together with the fact that the same range of rate change is covered at a much higher concentration of sodium than magnesium. For magnesium, the rate constant changes proportionally to the third power of the ion concentration, and for sodium it changes approximately as the sixth power.

Adams suggests that the lower reaction rates are due to the formation of a complex between phage and magnesium which is much more stable.

It is interesting that if $\Delta S^{\ddagger}$ values are calculated for various concentrations of Mg^{++}, the values do not vary monotonically but rise from 35 entropy units (calories/mole/°C) to 90 at $10^{-5}M$, fall to 40 at 10^{-4}, and rise to about 90 for $10^{-3}M$ and broth. This lack of a regular behavior probably shows the inherent complexity of thermal inactivation. After all, every part of the virus, whether protein or nucleoprotein, is thermally sensitive, and, moreover, the virus unquestionably comprises many parts combined together by loose physical forces. The inactivation of the virus as regards its infectivity probably involves the change of specific structure of only one of these.

Under some circumstances, different units may inactivate first and so be the rate setting unit. It would appear as though varying the ionic concentration causes a shift from one sensitive unit to another. The consideration which determines the particular part is not an entropy but the actual rate at the chosen temperature. The curves of Fig. 4.5, if plotted for rate con-

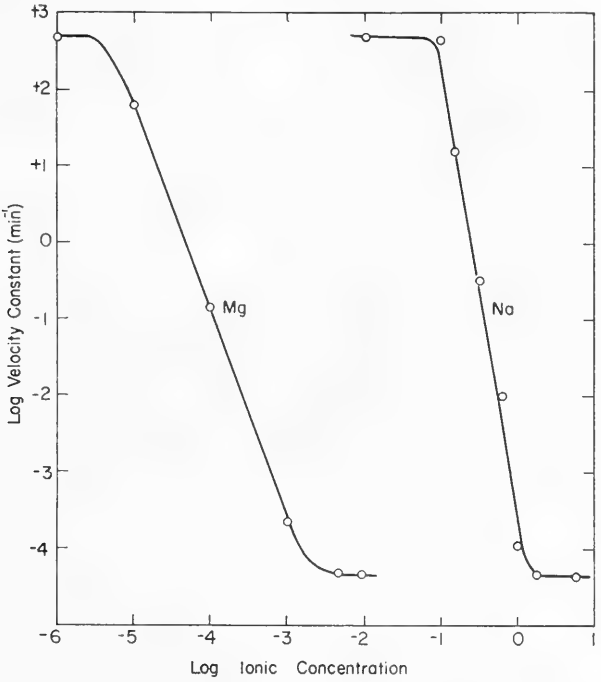

FIG. 4.5. Effect of ionic strength on the rate constant for inactivation of T-5 phage. Data due to Adams (1949).

stants at 30° C, show appreciable variation from what is shown, notably in a more rapid dependence on ion concentration.

It must therefore be remembered that, although thermal-inactivation studies undoubtedly contain valuable information about viruses, the unraveling of the various factors involved will take time.

It is of interest that thermal resistance can appear as a mutation. Adams and Lark (1950) have shown that a mutant

form, which transmits hereditarily, is much more stable at low salt concentrations. This mutant adsorbs in the same way and has the same serological affinity and the same latent period as the wild type. At high broth concentrations, the wild type and the mutant are the same.

The latent period and the burst size of T-5 are both modified by citrate, which shows that a part of the virus which is not concerned with duplication itself, but rather with rate, exists. This follows the same line as has been mentioned for latent-period increase due to X-ray and deuteron bombardment.

The thermal inactivation of four bacteriophages has been studied by Marjorie Reaume and the author (Pollard and Reaume, 1951). The inactivation, both wet and dry, was studied with results which can be seen from Table 4.1. The striking fact is that in the dry state the entropy is very greatly reduced and is actually very nearly zero. The entropy does not seem to be related to the size of the virus either in the dry or wet state. This means that the virus inactivation is taking place in relatively small regions and is again indicative of structure and function in the virus. The contrasting character of inactivation and denaturation calls attention to a minimum of two different parts of the structure. If it were true that thermal inactivation showed characteristic behavior for different substances, then the figures of Table 4.1 could be used to determine which proteins, nucleoproteins, etc., were involved in the activity measured. Unfortunately this is only partly true. Possibly in the dry state, where very little work has been done, it is possible to characterize an enzyme or protein by its inactivation constants. In the wet state, many factors must first be considered.

Inactivation as a Function of *p*H

If the percent infectivity of T-1 phage, or of TMV, remaining after a fixed time of exposure to thermal action, is plotted against the *p*H, the type of result shown in Fig. 4.6 is obtained. There is a region of relative stability with rather sharp edges. Steinhardt (1937) has shown that for pepsin the rate of denaturation varies as the cube of the hydrogen-ion concentration, and concludes

that the changing of three ionically bonded groups is necessary for the unfolding, and hence denaturation, of pepsin. The T-1 inactivation-reaction constant varies more slowly, more nearly as the first power, and so perhaps corresponds to the changing of a single ionically bonded group. Much better data is really needed for this sort of analysis.

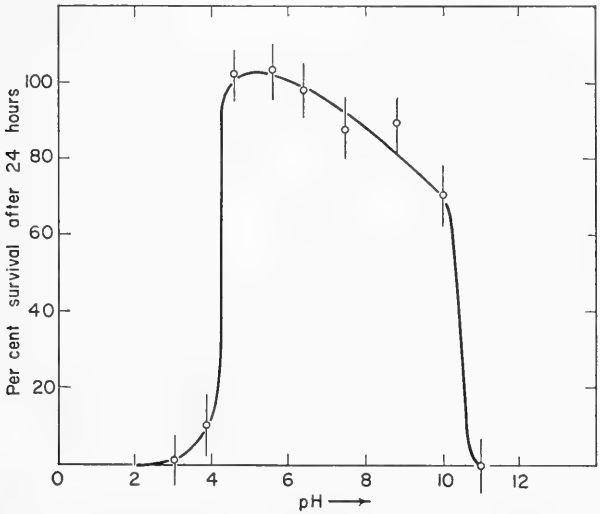

FIG. 4.6. Percent survival of T-1 phage after exposure to different pH values for 24 hr.

PRESSURE EFFECTS ON THERMAL INACTIVATION

A systematic study of the effects of pressure on microorganisms has been made by Johnson. By way of illustration of a case which behaves regularly and is susceptible of analysis in terms of the Eyring theory we show, in Fig. 4.7, data taken by Foster, Johnson, and Miller (1949) on T-5. The inactivation in the presence of a small concentration of Mg^{++} ions obeys first-order kinetics, and it can be seen that as the pressure is increased the rate of inactivation is lessened. This corresponds to a positive change, or an increase in volume, to reach the activated state. The value of $\Delta V^{\ddagger}$ found is 113 cm^3/mole, or 1.87×10^{-22} cm^3/virus particle. The total volume of the virus is about

3×10^{-16} cm^3, so this corresponds to a percent volume change of 6.1×10^{-5}. Such a small change, seen as part of the whole virus, seems too minute to be effective. It is more likely to be thought of as a volume change in one sensitive molecule of molecular

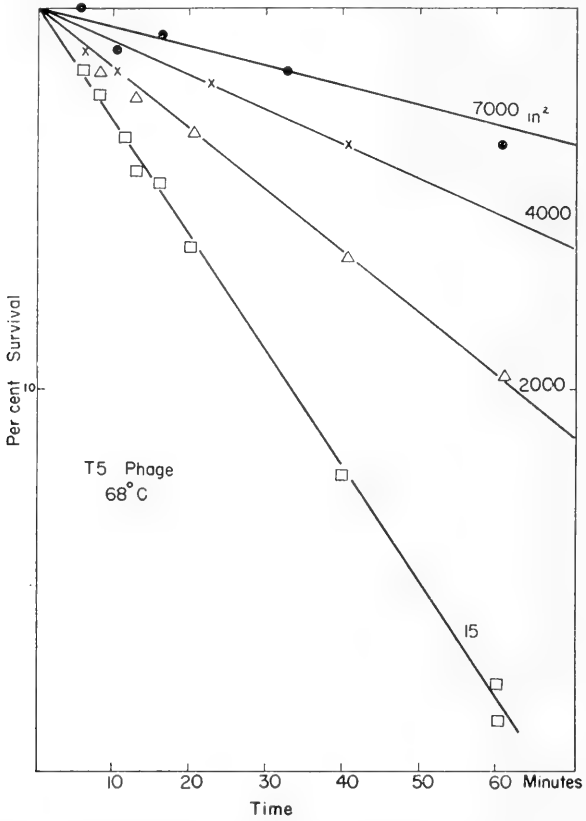

FIG. 4.7. Effect of pressure on the inactivation of T-5 according to measurements of Foster, Johnson, and Miller (1949). Pressure reduces bond lengths and gives an increased stability.

weight, say, 100,000 for which the volume change is about one-tenth of one percent or so.

The figures obtained by Foster, Johnson, and Miller for $\Delta H^{\ddagger}$ and $\Delta S^{\ddagger}$ do not agree with previously quoted values obtained by Adams. Whereas this may well be due to the difference in salt

content of the broth used, it is quite possible that the effect of pressure is to cause a change in $\Delta V^{\ddagger}$ such that different parts of the virus, with normally slower rate constants, become the rate determining factor. It must never be lost sight of, in this kind of work, that a virus is not a single molecule but, at best, a molecular aggregation.

A rather different approach from the statistical-thermodynamical reasoning of the Eyring theory may be more significant. It seems very likely that the nucleoproteins of viruses have very high changes in volume under pressure. This means that a fair fraction of the bonds are capable of changing their average spacing. If the energy of binding of such bonds is strongly distance dependent, the increased proximity of the bound groupings may increase the stability of the molecule as regards thermal agitation. The positive $\Delta V^{\ddagger}$, which means that the molecule must expand to inactivate, is thus to be linked with the idea that the molecule with high $\Delta V^{\ddagger}$ contains bonds which are quite distance dependent.

Since the above measurements were made on infectivity, which perhaps is related to nucleoprotein, a completely different set of values would be expected for change in serological affinity, which is related to the surface protein. Studies which could be used to measure $\Delta V^{\ddagger}$ for such treatment have not yet been made.

Conclusions from Thermal-Inactivation Studies

Virus inactivation seems to follow first-order reaction kinetics with an energy of activation in the dry state of around 1 ev. The constants in the wet state indicate that higher energy barriers have to be overcome, so that tighter binding of the whole structure holds in the wet state. This is at the expense of a high, positive entropy of activation, corresponding to the binding of considerable water, with consequent reduction in possible degrees of freedom, and renders the virus quite susceptible to inactivation if the temperatures are high enough. Thus, viruses seem to be stable when dry if the drying process can be conducted so as to retain the virus structure intact, but the temperature must be kept low in many cases or the virus slowly gets

taken across the low potential barrier. On the other hand, wet viruses are highly stable at low temperatures but in a small temperature range may become inactivated very quickly.

The high entropies of activation associated with the denaturation of proteins are not necessarily found for virus inactivation unless it is carried out at high temperatures. This fact will be seen to be very significant in studies on the serological behavior of viruses, and it turns out that serological affinity follows denaturation behavior in many cases. Thus if a virus is inactivated rapidly at high temperatures, the virus protein is almost sure to be denatured, with consequent damage to the antigens. If it is slowly inactivated at medium temperatures over a period of days, there is good reason to believe that great loss of infectivity can occur with little or no loss in ability to combine serologically.

Many more studies of thermal inactivation need to be made. Thus all the complex of properties studied by Watson for T-2 bacteriophage and described in Chapter 3 can also be studied for thermal inactivation. Actually, practically none of this rewarding work has been done; this chapter is accordingly short. Further mention of thermal studies in regard to the surface properties of viruses are contained in the next chapter.

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CHAPTER FIVE

THE SURFACE OF VIRUSES

Viruses are perhaps the smallest functioning objects in nature. They are in the realm of colloids, and yet they have an internal structure and are able to use this internal structure to multiply when in a host. Their small size greatly exaggerates the relative importance of the virus surface. In a human being, the ratio of surface to mass is about 0.3 cm^2/gm, whereas in southern bean mosaic virus it is about 3-million cm^2/gm, or 10-million times larger. A human being functions considerably by reason of his surface, and so a virus must do so much more.

So great is this surface-to-mass ratio that it is impossible to conceive of a virus maintaining any kind of metabolism outside a host. If the remarkable relation between surface area and internal metabolism [as, for example, measured by respiration studies (see Brody, 1945)] is extended down to viruses, the respiratory rate is such that a gram of virus would require an energy turnover 20 times that of a normal human being. This is much too high to be going on continuously, and so this very simple consideration indicates that outside the host a virus can be treated as nonliving. Inside the host it is quite different. This is a striking illustration of the importance of environment.

The small size of a virus also influences the vapor pressure of water at its surface. If p' is the vapor pressure at a surface of radius r, and p is the ambient vapor pressure, then, for a liquid of density ρ, molecular weight M, and surface tension γ, the relation holding is (Adam, 1941, p. 14)

$$RT \ln \frac{p'}{p} = \frac{2\gamma M}{r\rho} \tag{5.1}$$

For southern bean mosaic virus $p'/p = 1.08$, so the vapor pressure is 8% higher than ambient. A virus, therefore, will tend to dry even in the presence of moderately high vapor pressures. It is essentially certain that all the water held by a virus is bound by some kind of moderately strong force not (necessarily) as strong as a covalent bond but of the order of a hydrogen bond in strength.

Surface Functions of Viruses

Many of the known properties of viruses are due to their surface. Those of great importance are now listed and briefly described.

Adsorption. This is best understood for bacterial viruses but is almost certainly a factor for all viruses, with the possible exception of plant viruses. The facts known about adsorption for bacterial viruses will be considered in more detail later in the chapter.

Serological Behavior. In some manner, the virus protein is able to condition the formation of antibodies in the blood serum of animals. These antibodies are then able to combine specifically with the surface virus proteins. This can be studied directly by measuring the precipitate formed, using various means for estimating small amounts of precipitate. Indirect means of various kinds have been devised, of which the most notable is neutralization of infectivity, by which the failure of a preparation to be infective at a certain dilution is taken as evidence that specific combination with antibody has taken place. Another indirect method is complement fixation, which is a remarkably sensitive technique embodying two separate serological processes. Sheep red cells can be dissolved by the joint presence of a thermally unstable substance present in normal serum, *complement*, and a specific antibody produced in rabbits after injection with sheep red cells. Complement is removed from serum by the combination of an antigen and an antibody. When complement is removed, the red cells are not dissolved. So the presence of specific combination between virus and antibody is inferred from the absence of complement, or the lack of dis-

solution of sheep red cells. This is quite a sensitive, but laborious, method.

The recent discovery that long polypeptides form a not highly specific and reversible combination with viruses has added a secondary aspect to serological work. The polypeptides are under better control as regards size and length than are antibodies and so can be used in different classes of experiment.

Hemagglutination. Certain animal viruses agglutinate red cells in a manner which is relatively simple to study. Hemagglutination may be followed by *elution*, wherein the virus releases itself from the red cells. Both properties are surface properties and both can be studied.

Adsorption

Although by far the greatest degree of study has gone into virus serology, it is simplest to consider the nature of adsorption because this forms the best introduction to the study of virus surfaces.

Bacterial viruses lend themselves very simply to adsorption studies. The virus and bacteria are mixed so that a known amount of virus is initially present and there is an excess of bacteria. After a certain time, which is usually varied, the mixture is spun in an ordinary laboratory centrifuge and the supernatant examined for virus. Any change in the amount of virus is ascribed to adsorption on the bacteria. The results of this type of experiment show that the rate of adsorption is proportional to the concentration of bacteria, B, and to the concentration of unattached virus, U, so that

$$-\frac{dU}{dt} = k_2 UB$$

which, on integration, gives the familiar relation

$$\ln \frac{U}{U_0} = k_2 Bt \tag{5.2}$$

Measurements of U/U_0, the percent virus left unattached as a function of time, thus enable k_2 to be measured if B is known.

The values of k_2 so measured are of the order of 5×10^{-11} cm^3/ sec. Krueger (1931) showed that adsorption to live and dead bacteria followed the above relation and measured k_2 for staphylococcus phage. Schlesinger (1932) pointed out that if the collision rate expected from Brownian movement of the bacterium and the virus were the only agent responsible for this rate, the value of k_2 calculated is about what is found. Delbrück (1940) assumes that because the virus is removed by adsorption near the surface of a spherical bacterium, radius a, there exists a concentration function $C(r)$, where

$$C(r) = C_\infty \left(1 - \frac{a}{r}\right)$$

where r is the distance from the center of the bacterium, and C_∞ is the concentration at infinite distance, i.e., the average concentration. There will then be a rate of flow, F, on to the bacterium given by the concentration gradient, $\partial C / \partial r$, times the area, $4\pi r^2$, times the diffusion constant, D, so that

$$F = 4\pi r^2 \frac{\partial C}{\partial r} = C_\infty 4\pi D a$$

Since $F = k_{\max} C_\infty$, we have $k_{\max} = 4\pi D a$, where $k_{\max}$ is the maximal attachment constant.

The fact that k_2 is so close to this figure argues that attachment occurs every time, that it does not depend on the orientation of the virus, and so that the whole virus surface, or at least an equally distributed pattern of small units, is involved in this attachment. Alternatively, an attraction of a specific charge must occur.

A great clarification of the nature of this process resulted from the very direct experiments of Puck, Garen, and Cline (1951). In studying values of k_2 by the method outlined above, they found that although T-1 and T-3 phages fitted the rapid adsorption pattern, T-2 and T-4 did not until NaCl was added. This feature of virus attachment had been discovered by Hershey (1946). Cherry and Watson (1949) observed that the lysis of *Streptococcus lactis* by a phage in the presence of $MgSO_4$ showed

a maximum at $0.007M$, of $CaCl_2$ at $0.02M$, and of KCl at $0.02M$ concentrations. They associated this with the adsorption process. Puck, Garen, and Cline then investigated the rate constant for the attachment of T-1 as a function of the salt concentration for NaCl and $CaCl_2$. Their results are shown in Fig.

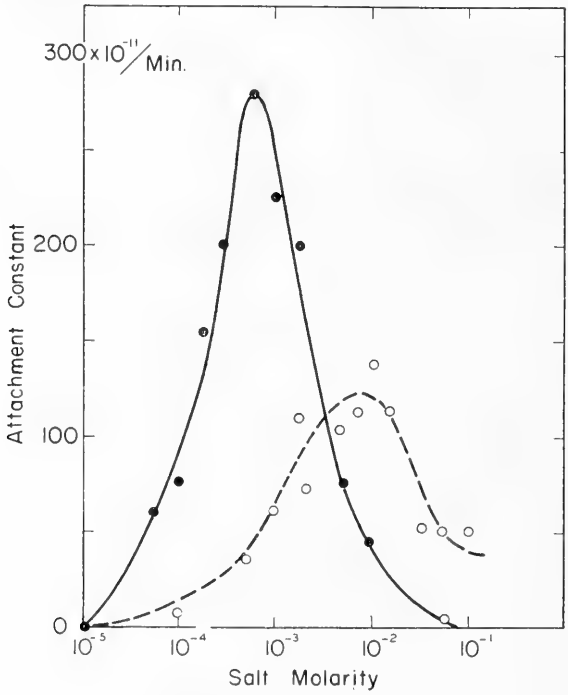

FIG. 5.1. Variation of the velocity constant for attachment of T-1 phage to *E. coli* with salt concentration, as measured by Puck, Garen, and Cline (1951). These results show the strong effect of the ions in the medium on the attachment.

5.1. There is a rapid rise in attachment for the divalent ions and a similar rise at higher concentration for monovalent ions. Excess of either kind of ion diminishes the attachment.

Further experiments showed that there is no lag period following the addition of divalent ions. The rapid attachment of virus is very striking in such a case. Also the reaction constant, while increasing from 2° C to a maximum at 37° C, does

not change very greatly. Part of the change is certainly due to the viscosity change of water. The virus is not firmly bound *at first*, and changes in the salt concentration can cause elution of the virus. This was shown by Garen and Puck (1951) who found that after a 10-min adsorption process, which gave a 98% attachment at 3° C, 43% could be eluted by raising the salt concentration. At 37° the amount that could be eluted was very much less.

By studying the reversible attachment at low temperatures and the irreversible attachment which occurs as the temperature is increased, the following picture of virus attachment emerges. The first process of attachment is electrostatic in character. It arises because a certain specific, charged grouping on the virus matches a specific, similar grouping on the bacterium surface. Such charges on the surface of colloids are well known. The charges in this case are controlled by the heredity of the virus and the bacterium. In the absence of any ions in the solution, we can suppose these charges to be alike, so that there is repulsion. Figure 5.2, taken from Puck, Garen, and Cline (1951), shows the kind of process. If, now, divalent ions are added, they can attach to the surface charges, and will do so in a preferential order. Suppose they first attach to the virus and not to the bacterium. Then the result will be a set of opposite charges, *in the correct pattern*, on the surface of the virus. The resulting attraction to the bacterium is then very strong. Quite simple considerations indicate that four charges which were accurately matched by four equal opposite charges can produce, at 10 Å, a force of 10^{-5} dyne/virus. At 50,000 g in an ultracentrifuge, the force per virus is only 10^{-12} dyne. The effect is thus 10-million times more potent than quite strong sedimentation.

As the ionic concentration increases, the ions continue to fasten to surfaces until the less favored groups on the bacterium are covered. The result is now much the same as at first, and a net repulsion exists. The curves of Fig. 5.1 are thus nicely explained. The divalent ion causes attachment at a low molarity, and also covers all surfaces to give repulsion at a high molarity. When only one type of surface (e.g., virus) is covered, the attrac-

tion is very strong. Monovalent ions are never as effective, operate at higher concentrations, and again, in excess, cause repulsion.

These experiments and the definiteness of this idea for the first attachment phase are relatively new. A complete theory in terms of modern ideas of colloids and ionic atmospheres should be capable of predicting the shape of the curves of Fig.

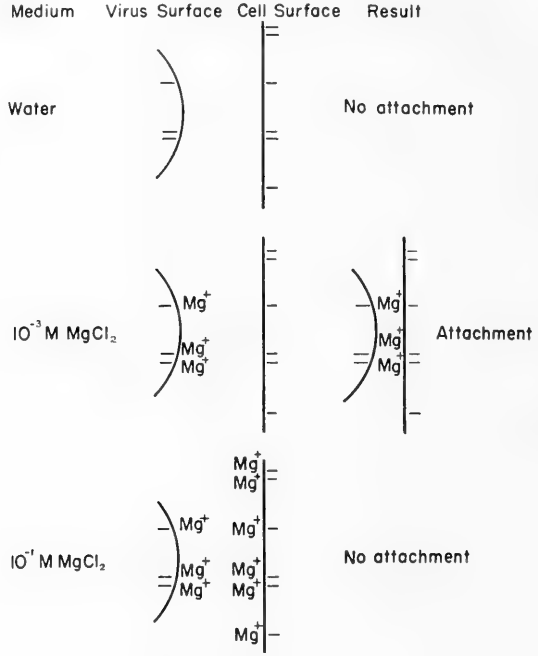

FIG. 5.2. Puck, Garen, and Cline's scheme for explaining the first phase of virus attachment.

5.1 and should then yield figures for actual charge distributions on the virus or the bacterium.

One important point which must be cleared up by such a theory is the factor which determines why ions should attach first to one or the other, virus or bacterium, and not equally to both. It seems probable that the final description of the process in terms of ionic-solution theory may be more complex than that just given.

One feature of this type of process is that, although specific attractions to identical surface distributions are very great, there is considerable attraction to any surface of opposite charge. The fact that viruses will attach to glass filters and other adsorbents has been known for some time. Delbrück (1940) found that reversible attachment of T-1 to a Jena glass filter was possible. Shepard and Woodend (1951) showed that T-2 phage can be adsorbed on to glass powder and celite filter aids and that the adsorption rapidly lessens above 10^{-2} molarity concentration. Puck, Garen, and Cline also demonstrated such nonspecific adsorption and, in addition, made the important point that a tryptophane-requiring mutant of T-2 would only attach to a glass filter in the presence of the needed concentration of tryptophane. The surface groupings in this case therefore require tryptophane to be present before they are formed.

Note that this electrostatic attraction is not temperature dependent except that the viscosity of the medium exerts a drag on the motion of the virus and this, being mainly water, changes rather slowly with temperature.

The second phase of attachment is described by Garen and Puck (1951). It was found by them that a part of the virus attachment to a bacterium is reversible. This reversible attachment does not kill the bacterium, takes place predominantly at low temperatures, and, in the presence of Zn^{++} ion, is the *only* kind of attachment. Reversibility was established by dilution in excess NaCl, which carries the virus to the low attachment point as seen in Fig. 5.1. The question arises as to the nature of irreversible attachment, which at 37° C is over 90% of the total attachment. In studying this, they found that it is strongly temperature dependent, with a $\Delta H^{\ddagger}$ of 18,000 calories/mole; that ultraviolet light applied to the bacterium inhibits it; and that ions are necessary for it to take place. They conclude that this second process is enzymatic in character.

The whole picture is then as follows. Surface charges of like configuration and charge exist on both bacterium and virus. In the presence of ions, one or the other preferentially attracts ions, so that matching *opposite* charges now exist, with conse-

quent strong attraction. Once attraction has occurred and the virus and bacterium are in contact, an irreversible enzymatic process takes place which is temperature sensitive, and inhibited by Zn^{++} in the case of *E. coli*.

Some interesting deductions can be made from this story. In the first place, the ionic binding is so strong that the virus and bacterium should be tightly locked together. This being so, the fact that the enzymatic process is 90%, or more, effective at 37° C means that a large part of the bacterial surface must be substrate. Garen and Puck also made the observation that 4×10^7 atoms of zinc are absorbed per cell of *E. coli* in order to block the enzymatic step. *Assuming* that these are all on the surface of the *E. coli* and that the bacterium is a cylinder 1μ in diameter by 2μ long, the total surface available for distribution is roughly 6×10^8 Å^2. Since this is matched on the virus surface by an identical distribution, the specific groupings must occupy an area of 15 Å^2. This number is probably low because it is based on the assumption that all the zinc goes in the surface of the bacterium. Nevertheless, the indication is that there exists a small charge grouping that is similar to that on the bacterium. Associated with this is an enzymatic type of surface.

These powerful methods of study are just in their early phases. Quite detailed knowledge of the virus and bacterial surfaces can be expected to result.

Polypeptide Attachment

Some very remarkable work in which the combination of lysine polypeptides with tobacco mosaic virus has been studied is due to Stahmann, Graf, Patterson, Walker, and Watson (1951) and to Burger and Stahmann (1951). In the first of these papers, the formation of synthetic polypeptides from E-carbobenzoxy-α carboxy-L-lysine anhydride is described. The chain length can be varied and measured by finding the proportion of terminal α-amino nitrogen. An elementary analysis of one such polypeptide gave $C_{1,366}H_{1,754}N_{194}O_{294}$, which has a molecular weight of roughly 25,000.

They show that such a polypeptide can cause the inhibition

of infectivity of TMV as shown in Fig. 5.3. The inhibition of infectivity is reversible, as shown by diluting a 4:1 combination of virus and polypeptide. When diluted, the virus-polypeptide combination evidently loosens, as the infectivity returns to normal at low concentrations.

In the second paper, the process is studied in more detail with the electron microscope and with a quantitative method of

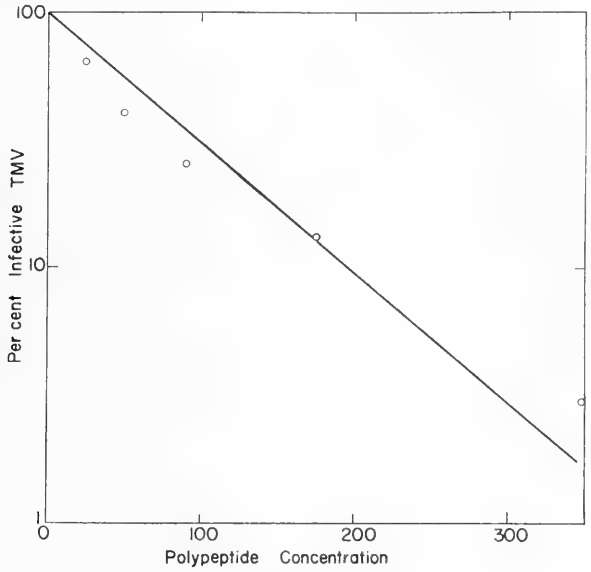

FIG. 5.3. Reduction of infectivity of TMV by lysine polypeptides in various concentrations according to the data of Stahmann, Graf, Patterson, Walker, and Watson (1951).

determining the amount of precipitate formed when virus and polypeptide are mixed. In addition, they carried out studies of the amount of virus-polypeptide precipitate formed when the *p*H is varied. Their results show that:

(*a*). The virus infectivity is reduced before a precipitate between virus and polypeptide is formed.

(*b*). No precipitation occurs at *p*H2 or *p*H10. This is the region in which both the virus and the protein carry a net charge of the same sign.

(*c*). Virus inhibition is a logarithmic function of the polypeptide concentration, a fact which is apparent from Fig. 5.3.

(*d*). The percent infectivity remaining is a falling logarithmic function of the chain length, or the number of lysine residues. The longer the chain, the more the inhibition of infectivity, but only logarithmically so.

(*e*). No precipitation of the virus occurs with free lysine.

They propose as an explanation that charged groups on the virus can combine with charged groups on the polypeptide which can then link to a second virus particle to cause aggregation. Aggregation does not occur at low and high *p*H. The reversibility of the process suggests that the binding is not extremely tight. For this reason, the longer the polypeptide chain the more charged bonds can be formed and the better the combination and inhibition.

In the electron microscope, the virus particles can be seen to be thicker and less uniform of surface. It is of interest that large aggregates can only form when the excess of polypeptide is not too great. It will be seen shortly that this is very much like the formation of precipitate with specific antibody.

These experiments offer a new technique in virus work. It may well be that inhibition of infectivity is general for all viruses, or specific to some. Inhibition of bacterial infectivity does take place (Burger and Stahmann, 1951), so some fairly general property of viruses, as, for example, the covering of the surface specific charges needed for attachment, may be involved.

Virus Serology

All kinds of viruses are excellent antigens. Plant viruses, in particular, are so good that if an infected plant is used to give infectious sap, and this is injected into a rabbit, the majority of the antibodies formed are attributable to the virus. This is partly related to the size of viruses, which are larger than more usual protein molecules, but it is probably also related in some way to the strong function a virus can play in a cell—even in a

cell which is not its host cell it is probably active, and one sign of this activity is the formation of antibodies.

Serological Techniques. Excellent accounts of these exist, and references are given at the end of the chapter. Two features of the technique are important. It is assumed that, as far as possible, an antiserum containing only the specific antibodies to the virus has been produced. This can be achieved by differential precipitation of all but the virus antiserum.

The two features of importance are (*a*) appropriate concentration relation between antibody and virus, and (*b*) assay of the amount of virus-antibody combination formed. The former is of importance because as the amount of antigen (virus) is increased relative to antibody the amount of precipitate increases, stays constant, and goes down. Therefore, working in the region of antibody excess must be established or ambiguities can result. Preliminary work has to be done to establish that the proper conditions hold. The second feature, the measurement of the amount of combination between virus and antibody, can be done in several ways. The simplest is to estimate by eye the amount of precipitate formed under favorable lighting conditions for different dilutions of virus and antiserum. A new technique due to Moorhead and Price (1953) raises the sensitivity of this method by using fresh, washed, sheep red cells as an indicator. Any precipitate between virus and antibody will keep the red cells from settling, and thus they form a convenient indicator for the presence of precipitate.

A quantitative method is to form the precipitate by incubation with antibody for the appropriate time (varying from 3 hr to 48 hr depending somewhat on the character of the test being made), centrifuge, remove the supernatant, dissolve the pellet in NaOH, and look for protein absorption at 2,750 Å in a spectrophotometer.

A third method is to employ the loss of infectivity of the virus as a result of antibody combination. This is particularly useful for bacterial viruses where the infectivity assay is direct and easy. Since the question of neutralization of infectivity is of

some importance, a short description of the major findings in this subject will be given.

Neutralization of Infectivity

If antibody is incubated for a standard (long) time with active virus, and the percent viable virus is plotted against the amount of antibody, three general types of curve are found. The first, which holds for T-2 phage (Hershey, Kalmanson, and Bronfenbrenner, 1943), is a semilogarithmic relation of the form

$$\ln \frac{n}{n_0} = -k_4 A$$

where n/n_0 is the surviving fraction, A is the amount of antibody, and k_4 is a constant. Viewing this process as due to a progressive covering of surface by antibody until the virus is inhibited, it is possible to estimate how many antibody molecules are needed for n/n_0 to be 37%, which represents a unity chance of virus inactivation. For T-2, the number found is 90.

The second type of curve shows an initial logarithmic behavior, but it flattens to a constant remaining activity. This is reminiscent of an equilibrium, but is not so. If further phage is added, precisely the same curve is obtained and the equilibrium point is not shifted. This type of relation holds for T-1. A simple explanation, given more for illustrating the type of process rather than claiming it to be true, is to suppose that two kinds of antigen exist. If one kind of antibody is predominently formed, and some cross reaction with the second antigen exists, then two rate constants would be found.

The third type is the same but is actually a reversible combination. This holds for influenza.

Surface Inactivation by Antibody

The figure of 90 molecules for T-2 inactivation is interpreted by supposing that a certain fraction of the virus surface is essential for its multiplication. This can be phrased by saying that some *bacterial* receptors exist on the virus which are impor-

tant, whereas *antibody* receptors are not. When any one bacterial receptor is covered, the virus is inactive.

The number of receptors can now be estimated. If it is assumed that the antibody molecules are of molecular weight 160,000, with a length of 235 Å and a diameter of 44 Å, and that they attach *end on*, we have:

Antibody attachment area $= \pi \times 22^2 \sim 1{,}500$ Å^2
Radius of T-2 phage = 350 Å
Surface area, ignoring tail, $= 4\pi \times 350^2 \sim 1.5 \times 10^6$ Å^2
So total area is equivalent to 1,000 molecules.

Now when 90 antibody molecules cover the appropriate spots, *one* bacterial receptor is just covered. This is one-eleventh of the whole surface and it can, therefore, be concluded that when one of 11 bacterial receptors are covered, the phage is inactive.

This fact of there being eleven receptors is of importance as it is another datum in the structure of viruses.

Serological Inactivation of Viruses

The techniques indicated in the two previous chapters should be applicable to the destruction of serological affinity. In fact, the technique of bombardment by ionizing radiation should be very suitable because we have just seen that something like a thousand antibody receptors must exist on a virus surface and so the size of each of these must be close to that of an enzyme molecule, which is inactivated by one primary ionization. The rather doubtful character of inactivation of infectivity, where it is questioned whether one primary ionization is sufficient, should not apply to the smaller molecules of the antibody receptors. We therefore describe some experiments in which the serological affinity of TMV, southern bean mosaic virus, and T-1 phage has been studied (Pollard and Dimond, 1952; Pollard and Jane Setlow, unpublished).

In the case of the two plant viruses, the technique used was to bombard virus preparations by deuterons and to assay for the amount of precipitate formed by centrifugation, pellet solution, and spectrophotometry. The results for TMV and

southern bean mosaic virus are shown in Fig. 5.4. The data for TMV are not too satisfactory because of a marked tendency for the virus to precipitate without addition of antibody after the very heavy irradiations necessary. This was allowed for

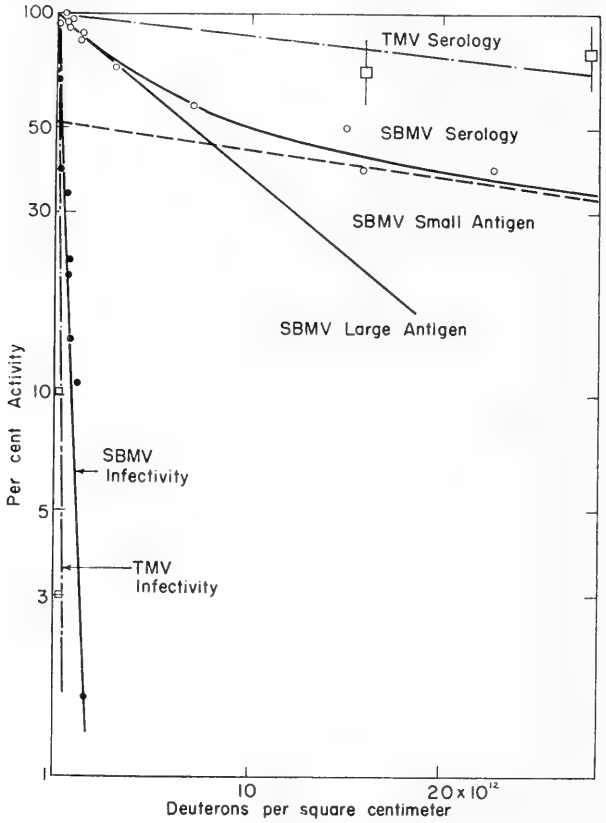

FIG. 5.4. Deuteron inactivation applied to the serological affinity of TMV and SBMV. The loss of activity is small compared to the loss of infectivity. The points for TMV fit best with a single antigen of surface area 1.5×10^{-14} cm^2, whereas for SBMV, two antigens of area 3×10^{-14} and 1.5×10^{-13} cm^2 are apparently present.

as best could be done, and the points are plotted. For SBMV the data are better and form two semilogarithmic lines which can be analyzed into two effective sensitive sizes. The values found are:

Virus	Area
TMV	1.5×10^{-14} cm^2
SBMV	3×10^{-14} cm^2 and 1.5×10^{-13} cm^2
T-1	1.5×10^{-13} cm^2

In the case of T-1 phage, similar results are found. The area per antigen is given in the table above.

These results are remarkable in that they indicate that the surface antigenic unit is quite small and also possibly multiple in type. The smallness of the unit gives rise to some speculation as to whether there may not be a rather limited number of possible types of antibody.

In the case of southern bean mosaic virus, the same ability to precipitate was measured after electron bombardment. The same type of double curve was obtained, again indicating two classes of antigen. It is possible, in principle, to apply the area and volume considerations of densely ionizing and sparsely ionizing particles. In the case of the small antigen, this can be done roughly, and the resulting sensitive volume is found to be about 7×10^{-21} cm^3 with a very roughly spherical shape. The molecular weight equivalent is about 6,000. The area of each antigen is about 3×10^{-14} cm^2, and of the whole dry virus it is 3.5×10^{-11} cm^2, so there are presumably 1,700 such small antigens. The large antigen cannot be analyzed so well, but by pushing the data a little, a molecular weight equivalent of 15,000 is obtained, with a roughly spherical shape and an area per antigen of roughly 1.5×10^{-13} cm^2. Assuming these cover the virus surface, there are about 200 large antigens.

The resulting picture of the virus is shown in Fig. 5.5. The large antigens alone are drawn in, but the subdivision of each into smaller units is also shown for one case.

The warnings contained in Chapter 1 must be repeated here. Such pictures and such models are necessarily inferential. For instance, the second antigen may not prove to be part of the virus itself. Other evidence should support them before they can be taken as valid. A certain amount of such evidence exists

and this will appear later. It is no surprise to anyone who has worked with the serological affinity of viruses that there are many surface antigens. The small-size antigen is perhaps more remarkable. The area of 3×10^{-14} cm^2, or 300 Å^2, was found by Hutchinson (1952) for the effective antigenic area of monolayers of bovine serum albumin. Quite small molecular units are, therefore, capable of specific combination. Probably four or

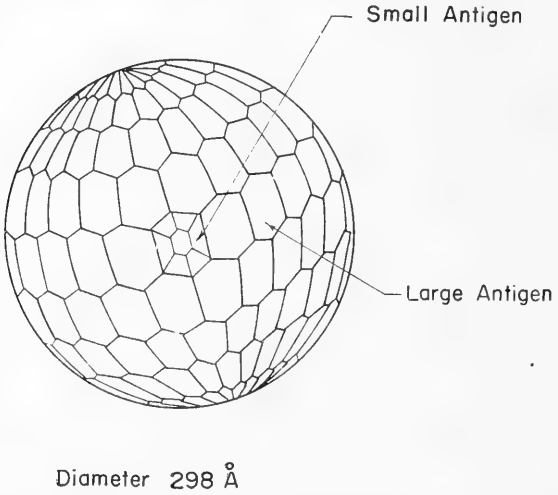

FIG. 5.5. Representation of the surface of southern bean mosaic virus as deduced from deuteron and electron bombardment and measurement of the serological affinity. The virus surface is composed of about 200 antigenic units of molecular weight about 15,000, and 1,200 subunits of molecular weight about 6,000.

five amino-acid side chains in a specifically repeated pattern, *throughout the virus surface*, form the combining antigenic units. Since there are only 20 amino acids, the possible combinations of four of these is not so enormous a number as the possible protein molecules. This may mean, as has been mentioned before, that a fairly limited number of specific group combinations on antibodies exist.

One byproduct of this type of work results. If the removal of serological affinity requires the inactivation of several hundred molecules, whereas the destruction of infectivity needs only a

few, it should be possible by deuteron bombardment, and nearly as well by electron bombardment, to produce an inactive virus which will stimulate antibody formation very nearly as efficiently as the active virus itself. This has been tried, quite independently of the above work, by Traub, Friedemann, Brasch, and Huber (1951). These workers have prepared a rabies vaccine by electron bombardment. The technique used is to harvest brains from mice injected intracerebrally with stock virus, waiting until 24 hr after the first symptoms developed. These were ground up, broth added, frozen in polyethylene bags, and exposed to 3-Mev electrons in the frozen condition on a solid CO_2 support. They were then thawed and tested for antigenicity. This was done by injecting at various dilutions into rats and then injecting challenge doses of the original virus preparation at various concentrations. The results are shown in the table.

Dose (10^6 rep)	Logarithm of infectivity titer of original	Logarithm of challenge virus control	Logarithm of virus after vaccine	Logarithm of protection
1.5	7	7	1.6	5.4
2.2	5.9	5.9	2.5	3.4
2.2	6.5	6.5	1.7	4.8
2.5	8.0	6.6	1.4	4.2
3.9	6.5	7.5	3.5	4.0
4.7	7.5	6.6	2.2	4.4

It can be seen that approximately 10,000 times as many original infectious virus particles are needed after such vaccination.

The amount of electron dose was found to diminish the logarithm of protection. If this fits a semilogarithmic relation (and the data are not complete enough to permit this deduction), then 26×10^6 rep are needed to cause a drop in the serological potency to 37%. This is 1.59×10^{19} primary ionizations/cm^3, so that the inactivation volume is $1/1.59 \times 10^{19}$, or roughly 6×10^{-20} cm^3, corresponding to an equivalent molecular weight

of about 45,000. This is somewhere near equivalent to the large antigen for SBMV.

This type of bombardment is probably a nearly equal mixture of indirect action due to free radicals and direct action due to primary ionization. The radicals act on the surface and many of them are needed for one inactivation. So indirect radiation action is a poor way to reduce infectivity and retain serological potency. For this reason, it is likely that the estimates made above give too large a size for the antigenic units.

There should be great practical application for dry, or well protected, electron bombardment of viruses. Deuteron bombardment should be even better.

Thermal Inactivation of Serological Affinity

The question as to whether infectivity and serological affinity inactivate thermally in the same way is clearly interesting. Bawden (1950, p. 253) gives figures for the loss of infectivity and of serological titer for potato virus X and bushy stunt virus after 10-min heating at *p*H 6 and different temperatures. The two viruses behave quite differently. Bushy stunt loses all its serological affinity between 80° C and 85° C, whereas the infectivity gradually diminishes over the range 50° C to 85° C. On the other hand, potato virus X loses its infectivity over a range from 59° C to 68° C and loses its serological affinity at about the same rate. Bawden points out that at 50° C, potato virus X loses its infectivity slowly without much serological loss. Probably the denaturation point is reached rapidly for virus X but not for bushy stunt.

Further studies, in which the logarithmic character of the loss of serological affinity is approximately demonstrated, have been made by the author and Dimond (1953) for southern bean mosaic virus and by the author and Jane Setlow (1953) for T-1 phage. Several curves for T-1 serological inactivation are shown in Fig. 5.6. These data were obtained by the technique of neutralization of infectivity. Inactivations were carried out, both wet and dry, with reasonable fit to a first-order reaction

kinetics, except at very low temperatures. Some indication of a second type of antigen was found below 80° C.

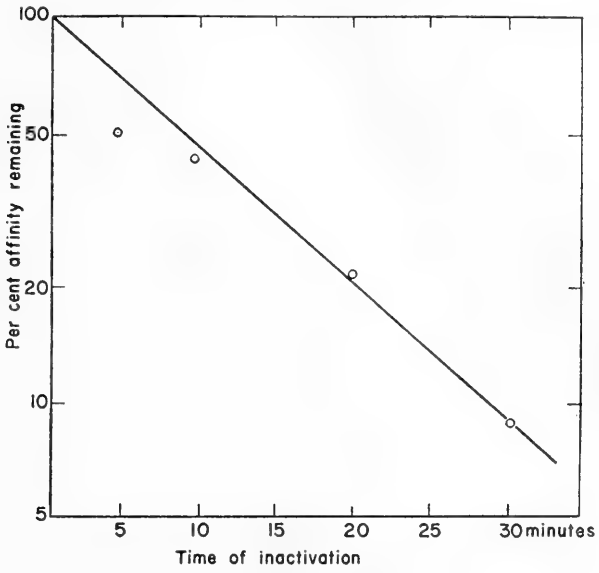

FIG. 5.6. Thermal action on the serological affinity of T-1 phage as measured by the neutralization of plaque formation.

These results can be analyzed as in the table. The values found for infectivity are given for reference.

REACTION CONSTANTS FOR T-1 INFECTIVITY AND SEROLOGY

	Infectivity		Serology	
Condition	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$	$\Delta H^{\ddagger}$	$\Delta S^{\ddagger}$
Dry	27,500	0	56,500	57
Wet	95,000	207	165,000	372

The results found by Dimond and the author for SBMV indicate that there are curved lines on this logarithmic scale. These can be analyzed into the sum of two lines and interpreted as the separate inactivation curves for two antigens. Approximately 60% of the antigenic activity has a rapidly changing reaction

constant corresponding to $\Delta H^{\ddagger} = 159{,}000$ and $\Delta S^{\ddagger} = 370$ (these figures are only approximate), whereas 40% has a slowly changing constant with $\Delta H^{\ddagger} = 17{,}400$ and $\Delta S = -22.5$ (again only roughly). These two types of inactivation are identified with the two antigens found from deuteron and electron bombardment, the large antigen corresponds to the slowly changing constant and the small to the rapidly changing one. It may be coincidence, but the values for T-1 phage agree rather well, both in target size and $\Delta H^{\ddagger}$, with SBMV. Clearly, many more such studies need to be made to see if this is a general type of antigen.

Hemagglutination

Some of the phenomena which can be so effectively studied in the interaction of phage and bacterium can be looked into by observing hemagglutination. This is a phenomenon discovered by Hirst (1942) which takes place for a fair variety of red cells and a moderate number of animal viruses, notably influenza, Newcastle disease, and mumps. Studies comparable to those of Puck, Garen, and Cline should be possible, although they have not yet been carried out. We are here concerned with two physical studies of hemagglutination, the work of Lauffer and Miller (1944) on influenza virus, and some recent work by Woese in the author's laboratory on the action of ionizing radiation on Newcastle disease virus.

In the experiments already quoted in Chapter 2, Lauffer and Miller showed that, for influenza virus, infectivity and refractive index move in the same way. The fact that this virus also possesses the property of agglutinating red blood cells enables a test to be made of the identity of the physical, infective, and agglutinating units. The calculated sedimentation rate from boundary measurement is compared with the agglutination activity above the barrier in the sedimentation cell with the results shown in the table. It can be seen that hemagglutination follows the boundary in a very satisfactory way. Agglutination is thus an inherent property of this virus. The figures for infectivity and nitrogen content are shown for comparison.

FRACTION REMAINING IN TOP COMPARTMENT
(From Lauffer and Miller, 1944)

Boundary measurement	Hemagglutination	Infectivity	Nitrogen content
100	100	100	100
45	57	57	65
35	35	—	39
34	37	—	39
25	23	20	17
15	12	15	—
0	2	—	—

The effect of exposure to temperature on hemagglutination was measured by Lauffer and Carnelly (1945). They found that the inactivation follows a form such that, at any one temperature, the reciprocal of the square root of the agglutinating activity is proportional to time. This corresponds to a reaction of the three-halves order, which is hard to interpret literally. Lauffer and Carnelly point out that there is probably some kinetic process complication, as, for example, multiple sensitivity of agglutination receptors. In any event, it is possible to characterize hemagglutination in this way even though it is somewhat empirical.

Using this basis of measurement, Lauffer and Scott (1946) measured the rate constants at various temperatures and concluded that, for the loss of hemagglutination, the value of $\Delta H^{\ddagger}$, the energy of activation, is 110,000. The entropy of activation cannot be calculated, as the reaction is not of simple order. This value for $\Delta H^{\ddagger}$ is considerably higher than the energy of activation for infectivity loss, which is 34,000 calories. This fits rather well with the behavior of serological affinity with regard to thermal action, and fits a protein figure rather than nucleoprotein inactivation kinetics.

Studies of Newcastle disease hemagglutination have been made by Woese (1953). The rates of loss of hemagglutinating ability in both the wet and dry state were studied. In the wet state, first-order kinetics were obeyed to a reasonable approximation. In the dry state, two reaction constants were clearly

observed. The relative proportions of slow-inactivating and fast-inactivating components are not, however, fixed, but as the temperature is increased, the fast reacting component increases in relative importance. Woese attributes this to an equilibrium between two forms of virus, both of which can become inactivated. He suggests that the equilibrium is conditioned by the amount of bound water remaining in the virus. The reaction statistics are given in the table.

Thermal Inactivation of NDV Hemagglutination

State of virus	$\Delta H^{\ddagger}$ (calories/mole)	$\Delta S^{\ddagger}$ (calories/mole/°C)
Wet	125,000	320
Dry (fast component)	23,000	−4.8
(slow component)	22,000	+0.2

In addition to these thermal studies, Woese has observed the effect of deuteron bombardment, in the dry state, on NDV hemagglutination. The radius of the unit which is responsible for this virus property lies between 40 Å and 50 Å and so has a molecular weight of the order of 300,000.

Nature of the Virus Surface

By way of a conclusion to this chapter, a brief description of the virus surface, as deduced from the work described, is now given. The surface is covered with many similar groupings, each carrying a charge. These groupings are doubtless of the familiar type of organic acid-base combination, and so acquire a charge in a manner dependent on the hydrogen-ion concentration. The number of such groupings is certainly large, of the order of thousands, and when a similar group of the opposite charge is presented near the virus, a very strong force pulls the two together.

These groups are probably part of, or close to, enzymatic elements, which can catalyse an irreversible reaction which may either bind the virus on to a cell or, in the case of elution from

red cells, may destroy the surface pattern responsible for adsorption in the first place.

Probably the enzymatic part is also related to the antigenicity of the virus, for there appear to be many surface antigens, of the order of 1,000 small units and several hundred large units. These units must have a great many common properties, although they are not known to be identical.

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CHAPTER SIX

ACTION OF ULTRAVIOLET LIGHT ON VIRUSES

The biological action of ultraviolet light has been the subject of much study. The side of this work which is of most interest here is ably summarized by McLaren (1949). An important pioneer in the field of ultraviolet effects on viruses and enzymes was Gates (1930). The more recently available purified virus preparations have enabled some important new contributions to this field to be made and there is, therefore, considerable material for this chapter. In its simplest terms, ultraviolet light gives information about the absorption of ring aromatic compounds, purines and pyrimidines, and the peptide bond. The former are associated with proteins; the purines and pyrimidines, with nucleic acids, and the peptide bond is related again to proteins. So a separation of protein and nucleic-acid absorption is possible, and this is one of the first functions of ultraviolet studies. More refined studies can tell something of the relative importance of aromatic amino acids in various kinds of function. An additional feature of importance is the quantum yield of a biological result, a number which can be related to both structure and purpose.

Molecular Absorption of Ultraviolet Light

It is in order to consider first the mechanism of the absorption of ultraviolet light. Light is electromagnetic radiation and so interacts with the electric and magnetic elements of molecules. In particular, it does so with oscillating electric dipoles, magnetic dipoles, and electric quadrupoles; but in actual fact the degree of interaction is only appreciable for electric dipoles. So, for all intents and purposes, we can say that light is only absorbed by

a molecule which has a method of motion which is equivalent to the proper electric dipole.

In the case of molecular absorption of ultraviolet light, the vibrations of atoms as a whole in the molecule are of far too low a frequency. Hence, the only suitable means of absorption is by a transition from one electronic state to another, which, as it primarily involves single electrons, has the proper frequency range. Inevitably associated with this electronic transition is some kind of vibration, and the combination of the two gives

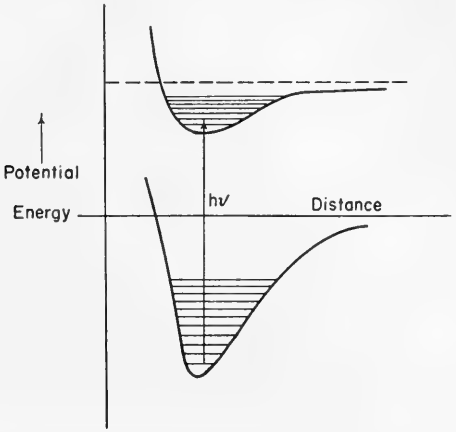


FIG. 6.1. Electronic absorption from one state of vibration to another in which an electron is in an excited state. This is the major process of ultraviolet absorption.

rise to an electronic-vibration *band*. Rotation is also possible, although in a large protein or nucleic-acid-like molecule it is probably not so important.

Using the familiar representation of potential hollows in which vibration can occur, such a transition is represented as in Fig. 6.1. Three important features need to be remembered. The first is that, inasmuch as an electronic transition changes the state of the electron cloud around an atom, there may be a great weakening of the bond joining this atom to a neighbor. The second is that the vibrational levels, and indeed all levels, are broadened by the thermal agitation and electric-field overlap of the near neighbors. For these reasons, ultraviolet absorption

by a large molecule cannot be anything like as selective a matter as absorption by an atom in free space. The third feature applies in solution. Since the presence of free ions in a solution can profoundly modify the stable electronic configuration of a compound (particularly in the organic acid-base, or "zwitterion" compounds), the absorption spectrum in solution may well depend on the number and kind of ions present.

In many cases the act of absorption can cause a bond to break. In a small molecule this causes dissociation. In a large molecule, however, the existing structure may be so strong that no actual atomic motion takes place and ultimately the energy is lost as radiation of some form, with consequent restoration of the bond. This process, known as the "cage-effect," or the Franck-Rabinowitsch effect, operates in the interior of a large molecule. The subsequent fate of the energy caught by the molecule in the act of absorption is complicated. In this respect the large-molecule constituents of viruses and the behavior of small crystals have much in common, so that virology has to look to developments and discoveries in solid state physics for an interpretation of some of the experimental findings in ultraviolet effects.

One process of energy transfer has been discussed for photosynthesis by Oppenheimer and Arnold (1950). They point out that the induction field of an excited bond may interact with another bond at a rather large distance and cause a transfer of energy without actually any radiation and absorption having taken place. This energy transfer process is similar to that of the "diffusion" of color centers in an alkali halide crystal, which has been experimentally studied by Apker and Taft (1950) and theoretically discussed by Heller (1951). Transfer of energy over distances as great as a micron are possible. This means that it is possible for energy to be transferred rapidly from any part of a virus to any other part.

At the surface of a large molecule, de-excitation can occur as a result of collisions with the solvent. Because of this, and because of the cage effect just mentioned, the denaturation of proteins by ultraviolet light is relatively inefficient.

Absorption by Some Definite Molecules

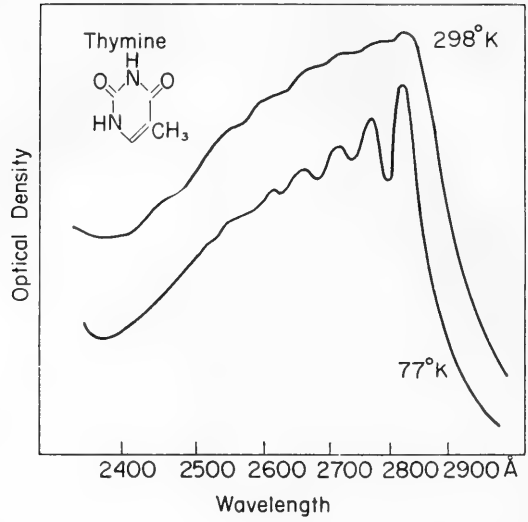
Absorption spectra of simple substances such as benzene vapor are very sharp. In solution, or in the liquid state, the lines are broadened. When a simple substitution is made on a molecule such as anthracene, the relatively simple spectrum becomes changed, in general because certain resonant electronic configurations are no longer possible.

Samples of various spectra are given in Fig. 6.2. An excellent review is given by Beavan and Holiday (1952). Of some interest is the sharpening of the absorption spectra of some organic compounds at low temperatures. This has been applied to molecules of biological interest by Sinsheimer, Scott, and Loofbourow (1950) and by Brown and Randall (1949). In general, one would expect a sharpening at low temperatures because of less molecular agitation. This is not found in practice. For example, purines show no sharpening, but pyrimidines definitely do. The absorption spectra of thymine and cytosine are shown in Fig. 6.2a and clearly have many distinct bands.

The effect of the nature of the solvent on the absorption of tryptophane and tyrosine can be seen in Fig. 6.2b where data due to Holiday (1936) are plotted. Although the general shape is not changed, the details of the peaks are considerably altered. The effect of substitution is shown in Fig. 6.2c where data of Jones (1947) are plotted for anthracene and anthraldehyde in ethanol. The substitution of the aldehyde group for the hydrogen removes many possible absorption modes, as can be seen from the figure.

The absorption spectrum of the pneumococcus transforming factor, a form of desoxyribose nucleic acid, taken by Fluke, is shown in Fig. 6.3. The absorption by the purines and pyrimidines causes the broad band at 2,600 Å. The rise at 2,200 Å is more doubtful.

In Fig. 6.4, the absorption spectrum of insulin, taken by Suprynowicz is shown. This is representative of protein absorption. A broad maximum occurs at 2,750 Å, but in this particular substance six peaks [previously reported by Beaven and Holiday



(a)

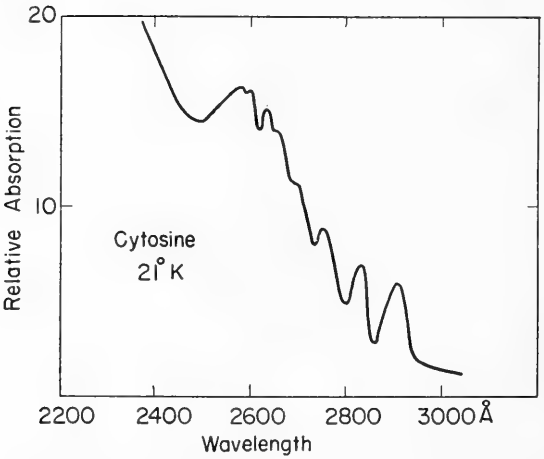
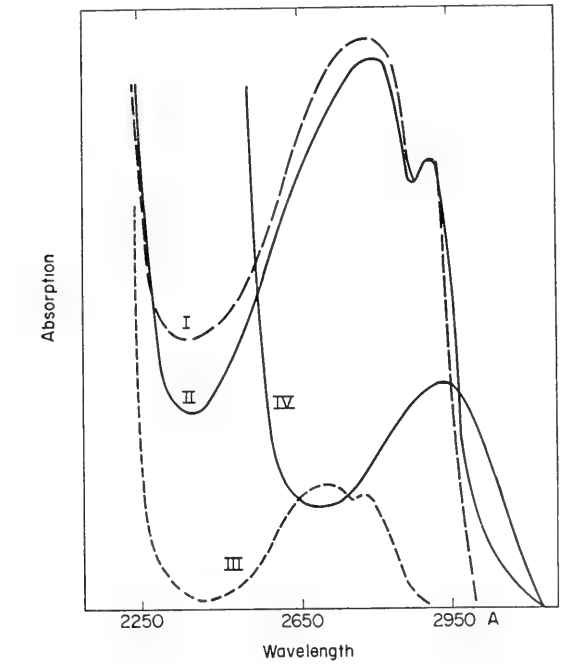
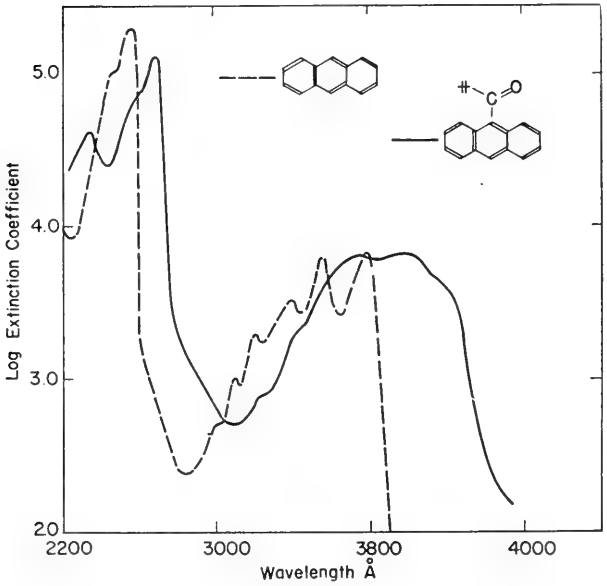


FIG. 6.2. (a) Absorption spectra of thymine and cytosine taken by Sinsheimer, Scott, and Loofbourow (1950) showing the sharpening observed at low temperatures. (b) Absorption spectra, taken by Holiday (1936), of tryptophane I and II and tyrosine III and IV in $N/10$HCl and $N/10$NaOH, respectively, showing the shift in character due to the shift in acidic or basic character of these substances. (c) Absorption spectra of anthracene and anthraldehyde, taken by Jones (1947), showing the removal of modes of absorption by the introduction of the aldehyde group.



(b)



(c)

FIG. 6.2. (*Continued.*)

(1950) at 2,535, 2,585, 2,645, 2,681, 2,755, and 2,831 Å] are found. These are presumably due to individual amino acids.

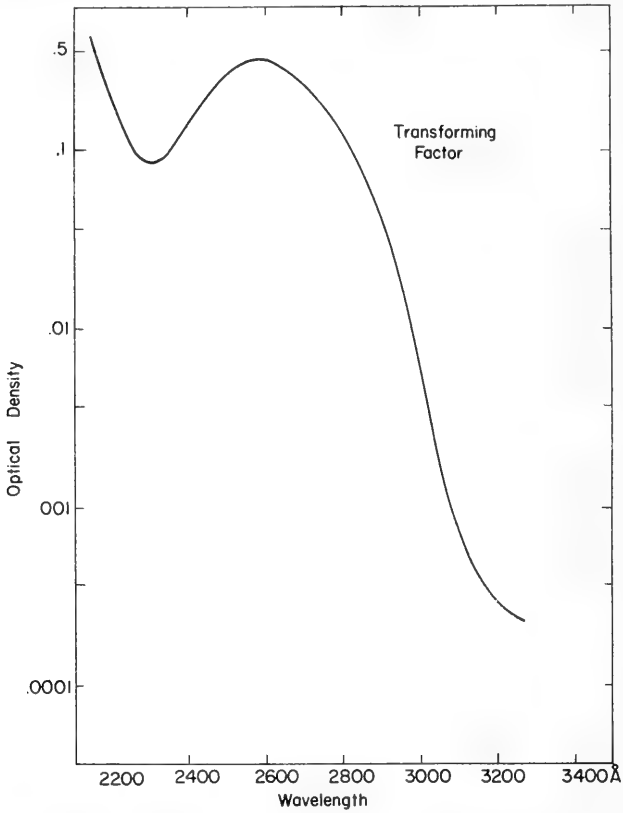


FIG. 6.3. Absorption spectrum of the pneumococcus transforming factor, a form of DNA, taken by Fluke. The broad band at 2,600 Å is due to the purines and pyrimidines. The origin of the rise at short wavelengths is indefinite.

THE ABSORPTION SPECTRUM OF TOBACCO MOSAIC VIRUS

Very careful work on tobacco mosaic virus was carried out by Butenandt, Friedrich-Freksa, Hartwig and Scheibe (1942). The absorption curve found is shown in Fig. 6.5b. It can be seen to have a rather definite structure. In Fig. 6.5a, the separate absorptions of tryptophane, ribonucleic acid, tyrosine, and phenylalanine are given. These can be assembled in the propor-

tions indicated in the lower curves of Fig. 6.5b. The result is not a complete synthesis of the observed absorption spectrum but is clearly rather close to it.

Some unpublished work by Brown and Randall (privately communicated by M. F. H. Wilkins) indicates that the absorption spectrum of TMV sharpens at low temperatures. The

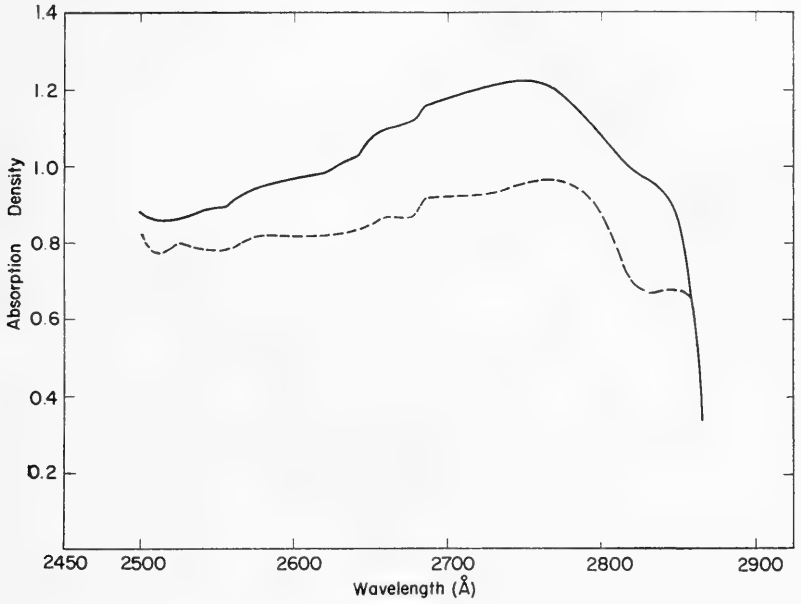


FIG. 6.4. Absorption spectrum of insulin, taken by Suprynowicz. The continuous line is at room temperature, and the dotted line at 80° K. Six peaks can be seen in a broad maximum at 2,750 Å. There is some sharpening at low temperatures.

absorption spectrum of TMV at room and liquid-nitrogen temperatures, as taken by Suprynowicz (1953), is shown in Fig. 6.6. The sharpening is quite apparent and can clearly be made use of in analyzing the internal structure of this virus. The wavelengths of the maxima observed are 2,578, 2,615, 2,644, 2,681, 2,811, and 2,905 Å. Of these, 2,644 and 2,681 coincide with two peaks found in insulin. TMV is predominantly protein and so it is not surprising that the absorption extends so far to the long-wave end.

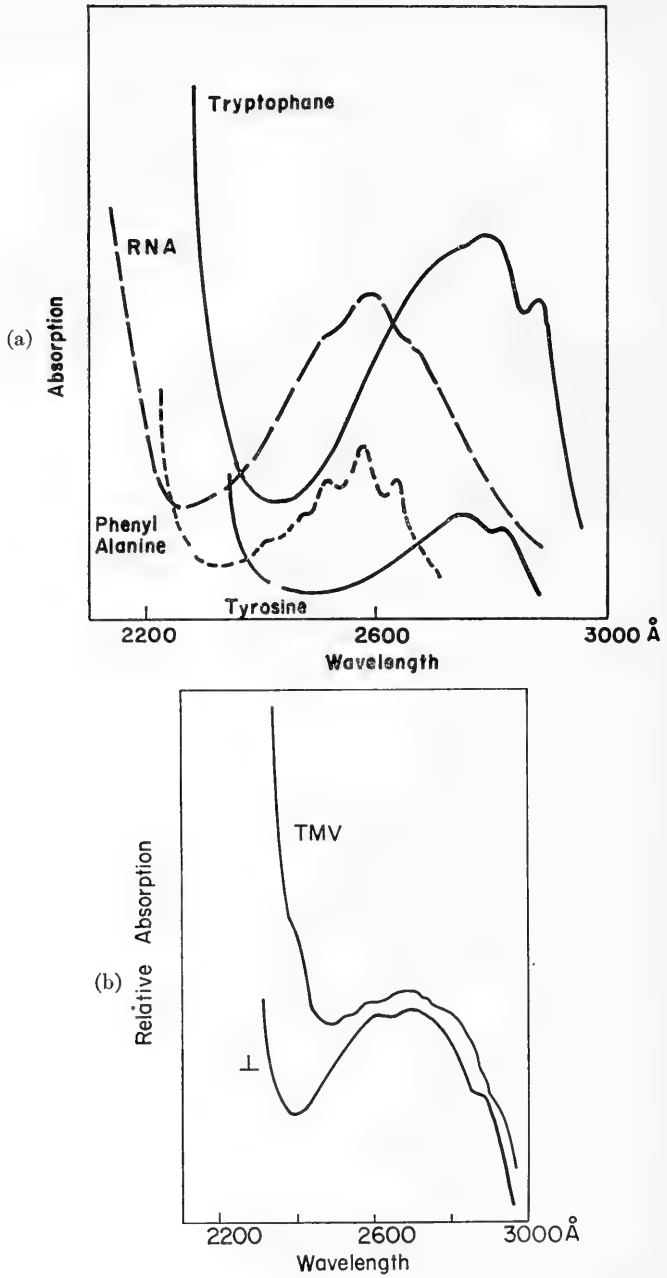


FIG. 6.5. (a) Absorption spectra of tryptophane, phenylalanine tyrosine, and RNA, taken by Butenandt, Friedrich-Freksa, Hartwig, and Scheibe (1942). (b) The absorption curve for TMV and, below, a line representing the synthesis of absorption by 4.5% tryptophane, 5% RNA, 3.8% tyrosine, and 6% phenylalanine. The general, but not detailed, characters are reproduced in this way. Data due to Butenandt *et al.*

The fact that tobacco mosaic virus consists of long, thin rods, and so can be oriented, means that the powerful tool of polarized ultraviolet absorption can be used. This was done by Butenandt, Friedrich-Freksa, Hartwig, and Scheibe. Tobacco mosaic virus is pressed into a fine capillary in a quartz tube and thereby acquires the orientation of the capillary. Light then passes through this capillary on to the slit of a spectrograph.

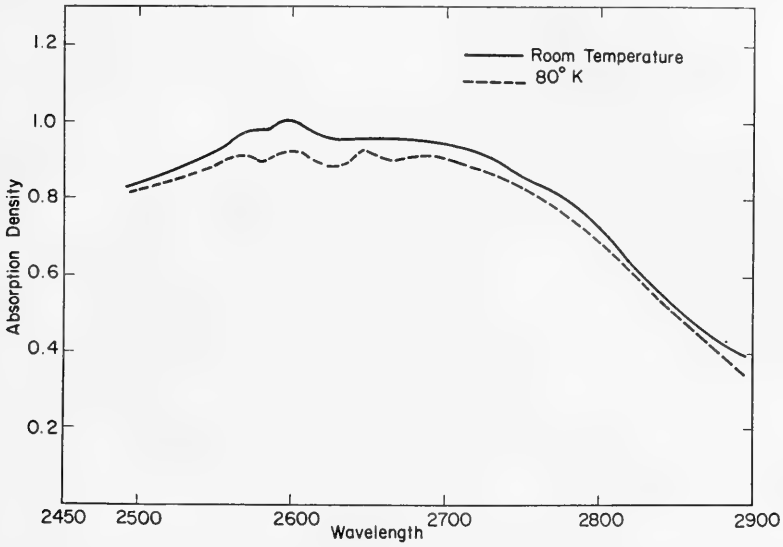


FIG. 6.6. Absorption spectrum of TMV at room and low temperatures, taken by Suprynowicz. Some sharpening is apparent, but no great increase in structure over what can be seen by careful examination of plates at room temperature.

At the exit end of the spectrograph is placed a sheet of crystalline quartz cut perpendicular to its optical axis and tilted at a slight angle so that the light having one plane of polarization is displaced to a different point than is light having a perpendicular plane of polarization. This occurs because the two classes of light have different refractive indices in the quartz. The spectrograph thus gives two images, one polarized parallel to the TMV in the capillary, and one perpendicular to it. The relative intensities of these, as a function of wavelength, are then measured.

The most striking result of this kind of study is the fact that the characteristic small absorption peak of tryptophane at 2,900 Å only shows for the case of light polarized perpendicular to the axis of the capillary, and hence of the virus. It is thus concluded that the aromatic rings of tryptophane are definitely oriented in the TMV particle. In a less clear way it can also be concluded that the nucleotides of ribosenucleic acid are also oriented. Butenandt concludes that the planes of the purine and pyrimidine rings are perpendicular to one another and perpendicular to the long axis of TMV. So also is the indole ring of tryptophane. Seed and Wilkins (1950) consider that orientation is necessary but that, in fact, it is parallel to the long axis of the virus.

More work along these lines would be of the utmost value.

Absorption Spectra of Other Viruses

The absorption spectra of southern bean mosaic virus and T-1 phage, as taken by Suprynowicz (1953), are shown in Fig. 6.7. These are both less full of character than is TMV and, in addition, show no sharpening of absorption at low temperatures. This last may partly be explained by the inability to orient the viruses in any way. The same kind of broad analysis into protein components and nucleic-acid components is possible. The rather definite increase in absorption below 2,300 Å is probably due to peptide bond absorption, which has been shown by Saidel and Goldfarb (1951) and by Setlow and Guild (1951) to be responsible for the rise in absorption of dipeptides below 2,300 Å. [Further interesting work on this has been reported by Goldfarb, Saidel, and Mosovich (1951) and Hamm and Platt (1952).]

Action Spectra

Biological action spectra are, in principle, very powerful methods of study of the internal structure and mode of operation of viruses. The technique consists of irradiating the virus, either dry or in solution, with monochromatic ultraviolet light and measuring the survival ratio for a known amount of incident

energy at that wavelength. For viruses, rather large amounts of light are necessary, so that either very intense ultraviolet sources or a monochromator with high aperture must be used. An inexpensive and quite adequate monochromator was designed and constructed by Fluke and Setlow (1951) using a large prism of distilled water enclosed with quartz plates and

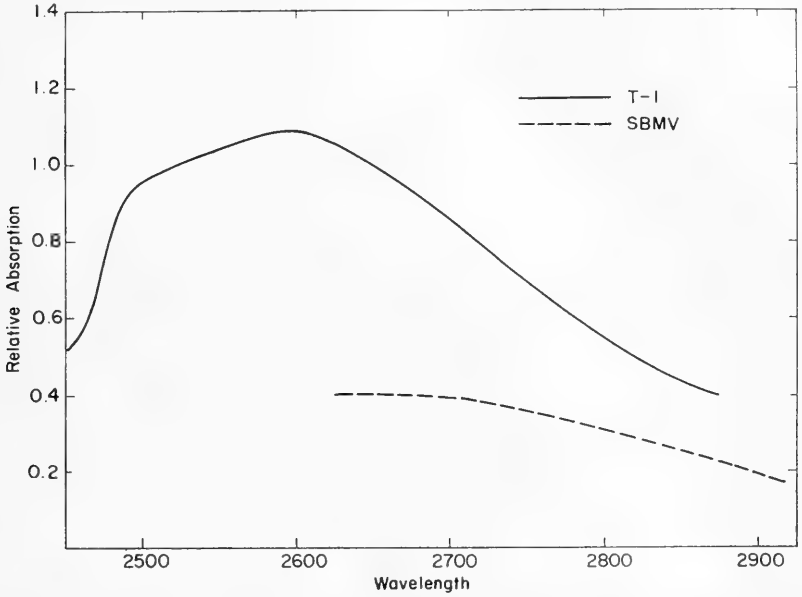


FIG. 6.7. Absorption spectra of T-1 phage and SBMV, taken by Suprynowicz. T-1 has a broad maximum around 2,600 Å. The absorption of SBMV extends quite far to the long-wavelength end.

two 8-in. astronomical mirrors coated with aluminum. The light source was a quartz mercury arc, and each line was isolated by an exit slit covering a bombardment chamber which could be employed either for stirred liquids or dry preparations. An important feature of this kind of work is the measurement of the radiant energy incident on the sample. For routine purposes this can be done with a calibrated photocell, but absolute measurements must be made now and again with a thermopile and sensitive galvanometer.

Careful studies have been made by Fluke (1953) on the action spectrum of T-1 phage, dry at room temperature, at liquid-nitrogen temperature, and wet at room temperature. In the dry state, it is found that the inactivation at any one wavelength follows the familiar relation

$$\ln n/n_0 = -aI$$

where n/n_0 is the survival ratio, I is the total energy incident on the specimen, and a is a constant depending on the amount of energy absorbed and the quantum yield in the process. In the wet state this relation sometimes holds, but in general it does not. The type of curve fits a so-called "multiple-hit" process. The general formula for an inactivation requiring m inactivating events for each of N necessary parts of a virus, with a probability a per unit total dose applied, is

$$\frac{n}{n_0} = 1 - \left\{1 - e^{-aI} \sum_{k=0}^{k=m-1} \frac{aI}{k!}\right\}^N$$

where k is an integer. (Timofeeff-Ressovsky and Zimmer, 1947; Zirkle, Marchbank, and Kuck, 1952.) When only one necessary part requires one inactivating event, we have the familiar survival curve $n/n_0 = e^{-aI}$. For two inactivating events, the two-hit curve is $n/n_0 = e^{-aI}(1 + aI)$, and so on. The multiplicity of hit is not definite and depends on the virus and the wavelength used. If an accurate survival-ratio curve is obtained, the number of hits can be ascertained and a measured.

For the relatively simple case of dry irradiated T-1, Fluke (1953) obtained the results shown in Fig. 6.8. The relative action on infectivity is plotted on a logarithmic scale versus the wavelength. This effectively means plotting a versus wavelength for survival. The virus was irradiated both at room temperature and liquid-nitrogen temperature. Slight but not very definitive differences are observed.

It is interesting that the action spectrum does not entirely parallel the absorption spectrum. Most noteworthy is the fact

that at the shorter wavelengths less effect on the virus is observed, whereas the absorption markedly rises. Since it is thought that this shorter-wavelength rise in absorption is due to the polypeptide absorption of proteins, it would seem as

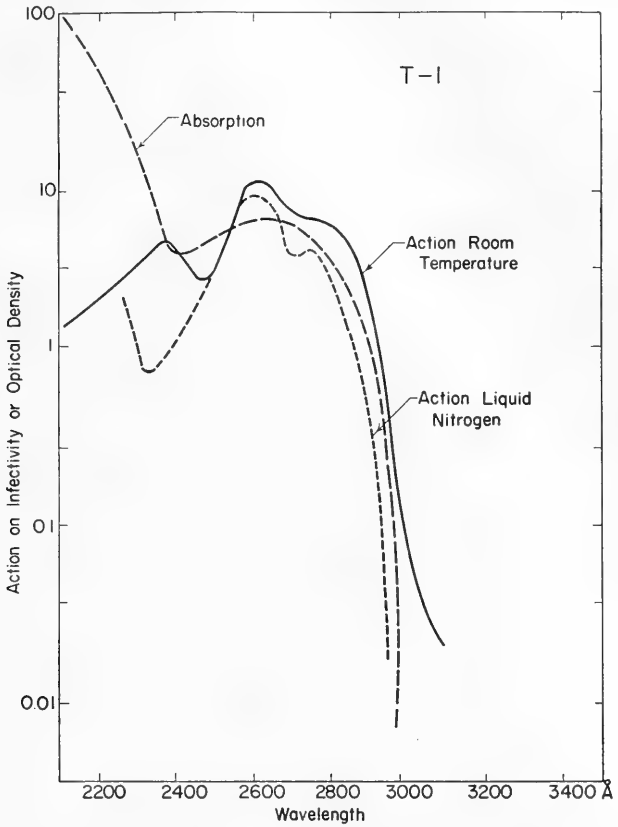


FIG. 6.8. Absorption and action spectra, taken by Fluke (1953), for T-1 phage. The action spectrum does not follow the absorption spectrum in detail, indicating that T-1 has a morphology, not all of which is concerned with survival. In particular, the quantum yield definitely falls at short wavelengths where polypeptide absorption occurs.

though either such protein absorption is unimportant or a considerable recovery takes place in bonds which have been excited in the polypeptide chain. On the other hand, absorption in the nucleotide or aromatic-amino-acid region seems to be quite

effective. The nucleotide absorption seems to be the most effective.

Such an action spectrum, although of very great value, represents only one step along a road that should lead to fundamental discoveries about viruses. From what has already been said, it is clear that the inactivation of a virus like T-1 is a complex process. It may be the result of over-lengthening of the latent period, which Luria (1944) has shown to occur following ultraviolet illumination, or it may be a lethal mutation in a genetic element, or possibly a destruction of specific surface groups. All of these various aspects of virus behavior have their separate action spectra, and only when a selection of these are available can the real power of the spectroscopic approach be apparent. This point is illustrated by some studies by Tamm and Fluke (1950) on influenza virus which are described below.

Infectivity and Hemagglutination Action Spectra of Influenza Virus

The infectivity and hemagglutinating ability of PR-8 influenza virus can both be readily studied. The former is measured by inoculating 10-day-old embryonated eggs and determining whether the inoculum results in a strong rise in virus in the egg due to active infection. The dilution at which this will occur is a measure of the activity of the virus. Hemagglutination is measured in a somewhat similar way by making twofold dilutions until one dilution fails to produce definite agglutination of red cells.

Tamm and Fluke employed monochromatic ultraviolet light of four wavelengths. The virus was irradiated in a gold-plated cell with quartz windows on two sides. Radiation intensity on both sides of the cell was measured by a phototube which was calibrated by means of a thermopile. Exposures were made for various times at known intensities.

The results are indicated in Fig. 6.9. The wavelength region covered is somewhat limited, notably in stopping short of the polypeptide absorption region, but it is clear that the maximum effect on infectivity is nearer to a nucleic-acid maximum, and is

different from the hemagglutination curve, which has a protein-like maximum. Perhaps most striking is the fact that, whereas the loss of infectivity followed an inactivation curve somewhere near a one-hit type, the hemagglutinating loss follows a strongly multi-hit type. Tamm and Fluke point out that

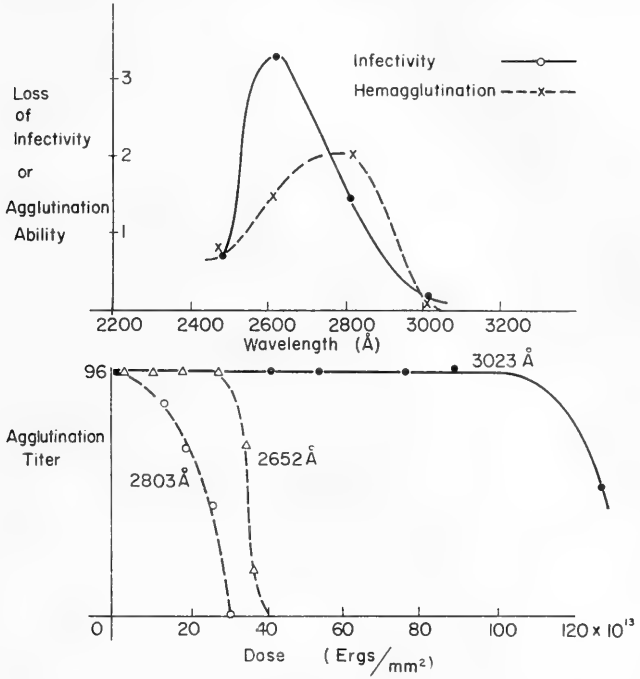


FIG. 6.9. Action spectra and dose-effect curves for influenza virus, as taken by Tamm and Fluke (1951). The hemagglutination maximum is clearly in the protein region, whereas the infectivity maximum is more nearly nucleic acid in character. The multi-hit character of loss of agglutinating ability can also be seen. It is both striking and variable.

this could be exploited for the preparation of vaccines, assuming that the serological behavior follows more the hemagglutination behavior. Ultraviolet inactivation has been successfully applied to produce rabies vaccine by Levinson, Milzer, Shaughnessy, Neal, and Oppenheimer (1945).

This work shows clearly that the study of action spectra does not yield a monotonous repetition of a nucleoprotein absorption

spectrum, but clearly enables the differentiation of various virus properties in terms of their chemical constitution and, possibly, their position in the virus. Thus, a virus having a sensitive region consisting of nucleic acid imbedded in protein will produce an absorption spectrum which is a composite of both protein and nucleic acid. The action spectrum, as regards the nucleic-acid part, will only show strong effect in the nucleic-acid absorbing region.

Work which could be extended to viruses has been carried out by Morowitz (1953) on *B. subtilis* spores. The lethal action spectrum and the action spectrum for two deficiency mutants were observed and compared with the absorption spectrum. Definite differences were observed.

Action Spectra for Different Viruses

A summary of earlier work on the action spectra of viruses was given by Hollaender and Oliphant (1944). Figure 6.10 shows a composite plot, adopted from their paper, for five viruses. The remarkably low sensitivity of Rous sarcoma virus at 2,470 Å and the high sensitivity of TMV at 2,300 Å are the most interesting features of these studies. It is also interesting that, although the absorption spectrum of TMV extends out to 2,900 Å, the quantum yield at that wavelength is very small.

This kind of experiment is difficult and laborious. However, it is clear that if the character shown in the five curves plotted is not due to experimental uncertainty, then virus action spectra should be useful in characterizing viruses.

Quantum Yield

One important datum in ultraviolet studies is the quantum yield for various kinds of inactivation processes. McLaren (1950) has made a careful study of quantum yields (ratio of number of molecules inactivated to number of photons absorbed) at 2,537 Å and has concluded that the larger the molecule (including viruses) the lower the quantum yield. Inverting the figure and talking of photons per inactivation, this number seems to be

roughly proportional to the surface area of the molecule. On rather slender evidence, McLaren (1951) has proposed that the ionic yields for the indirect action of ionizing radiation also obey this relation.

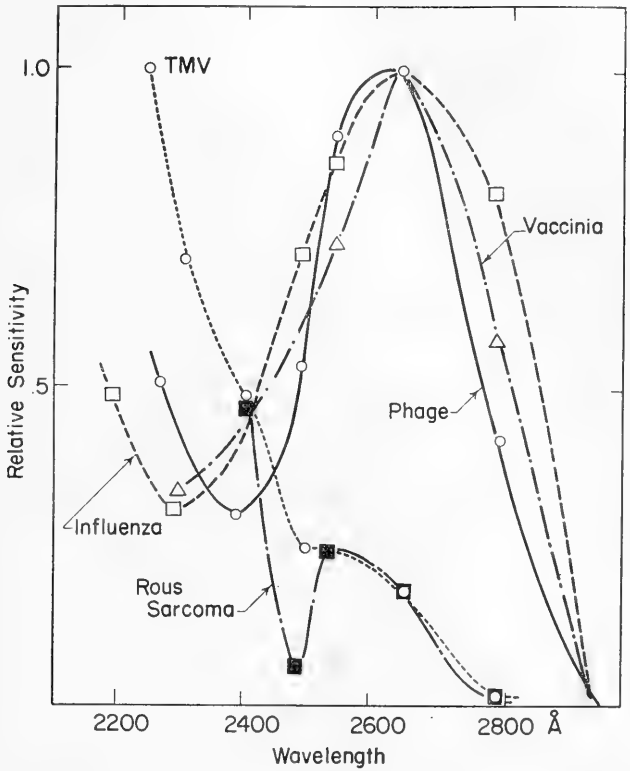


FIG. 6.10. Action spectra for five viruses, taken from Hollaender and Oliphant (1944). The phage acts on *Staphylococcus aureus*.

If this idea is right then the photons per inactivation should give some idea of the size of the unit being inactivated. Far too little data is at present available, but it represents an important region for future physical studies of viruses. The quantum yield for inactivation of TMV at 2,537 Å is 4.3×10^{-5} (McLaren, 1950), and for T-1 phage at 2,600 Å it is 3×10^{-4} (Fluke, 1953).

Multiplicity Reactivation after Ultraviolet Treatment

It was discovered by Luria (1947) that T-2, T-4, T-5, and T-6 phages, when inactivated by ultraviolet light and then caused to infect bacteria, multiply and show a much higher yield of still active phage than is the case if single infections alone are allowed. This shows that the virus is apparently partly inactivated, so that the single infection cannot quite achieve the process of multiplication, but yet has viral potency in some degree. If two viruses are used to infect a bacterium, there is now a chance that the residual potencies can combine to supplement each other in such a way that a whole virus results, with the usual consequences. This is spoken of as multiplicity reactivation and was originally analyzed in terms of a genetic recombination process (Luria, 1947; Luria and Dulbecco, 1949). This analysis depended rather critically on the one-hit nature of phage inactivation, and, since this does not necessarily hold, the precise conclusions may not be valid. Nevertheless, the numerical considerations are sufficiently interesting to be worth mentioning here in simplified form (following Luria, 1947).

Suppose a number, n, of essential units (not necessarily genetic) exist in each virus particle, and suppose r of these have been inactivated. Then the average "hits" per unit is r/n, and the probability of no inactivation is $e^{-r/n}$ per unit. The probability of at least one hit is $1 - e^{-r/n}$. If k particles are used to infect, the chance that a particular unit has been hit in every one of k particles is

$$(1 - e^{-r/n})^k$$

and the chance that one has *not* been hit is

$$1 - (1 - e^{-r/n})^k$$

The chance that a bacterium receives active representatives of all n units is y, where

$$y = \{1 - (1 - e^{-r/n})^k\}^n$$

This reasoning ignores the possibility that two hits may occur on one unit, and so has to be modified somewhat. This modifica-

tion was made by Luria and Dulbecco (1949). For low doses, where $r \ll n$, the value of y is

$$y = e^{-r^2/n}$$

assuming that only two phage particles are absorbed per bacterium. The value of r can be obtained from the dose-survival curve for single infection. At the 37% figure, $r = 1$, and, in general, if n/n_0 (the survival ratio) is measured, this is e^{-r}. With known values of r, infections which average two per bacterium can be made, and the value of the multiplicity reactivation can be found by comparison with the single-infection curve. The value of y is determined from the excess survival for double infection. The following figures are obtained.

Phage	r	$1/y$	n
T2, T2r	2.6	1	
	4.8	1.6	49
	7.7	3.5	47
	11.0	19	41
	14.3	93	45

The results, therefore, indicate that something like 50 essential units are present in each virus. Although this may be treated as no more than a figure to guide thought, it seems clear that the multiple character of virus structure is required by these experiments.

The action spectrum for this process would be of the greatest interest.

Although there is no necessary relation between this effect and the hypothetical structure deduced for T-1 phage from the lengthening of the latent period, it is interesting that a somewhat similar type of internal pattern is required for the two.

Photoreactivation of Bacteriophage

Further evidence that bacterial viruses are partly inactivated by ultraviolet light, and that in this condition they still possess some function, is found in the property of photoreactivation dis-

covered by Dulbecco (1949, 1950). When phages which have been inactivated by ultraviolet light (predominantly 2,537 Å), so that the plaque count is low, are irradiated *while in the bacterium* by very near ultraviolet light, the plaque count rises very definitely. This is the phenomenon of photoreactivation. Returning to the concept that the "enzyme" systems of phage can be damaged or reduced in number by ultraviolet light, there is evidently a condition in the bacterium which is favored by radiation, so that the handicapped phage can find the necessary precursors and survive to the first burst. The action spectrum for photoreactivation was measured by Dulbecco. The maximum occurs at 3,700 Å and some photoreactivation still occurs at 5,000 Å, well in the visible. Two classes of inactivated phage exist, one reactivatable, the other not. The fraction reactivatable is 0.7 for T-1, which is most reactivatable, and averages about 0.4.

Fluke (1951) has shown that the action spectrum for producing reactivatable and nonreactivatable phage is substantially the same. The major peak at 2,650 Å and the minor peak at 2,800 Å, as seen in Fig. 6.8, appear in much the same way. Hence, both nucleic-acid and protein absorption play a part in producing the damage, which is subsequently overcome by the photoreactivation.

Summary and Conclusions from Action Spectra

We give here a brief summary of the salient features of action spectra and one or two conclusions which can be drawn from them.

An electronic-vibration absorption is necessary.

Absorption in nucleic-acid bases or aromatic *side chains* is most efficient.

Polypeptide absorption is very inefficient.

The quantum yield is low. The number of photons needed is roughly proportional to the surface exposed.

The quantum-energy threshold is roughly three times the energy of the free-energy barrier for thermal inactivation.

Inactivation efficiencies are not very temperature dependent.

Strong sharpening is not apparent at low temperatures.

Since no appreciable low-temperature sharpening of the action spectrum occurs, it is likely that the type of absorption taking place involves transition to closely spaced levels—i.e., is near dissociation. Since many photons are absorbed to produce a one-hit (in some cases) inactivation, many excited bonds must lose energy and return to the original configuration. Since side-chain absorption is important, there is probably a severance of side-chain cross linkage which forms a part of the process of inactivation.

We wish to repeat that the study of virus action spectra is in its very early stages. As the varied kinds of virus inactivation are treated separately, the information can be used to give an inferred structure in terms of electromagnetic radiation, which can be used for comparison with inferences from ionizing radiation and thermal measurements.

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CHAPTER SEVEN

SONIC AND OSMOTIC EFFECTS ON VIRUSES

A sound wave is a compression-rarefaction wave which can travel in any material medium. It is possible to excite high-frequency sound waves very satisfactorily by using the piezoelectric properties of a quartz crystal. If a high-frequency electric field is applied to a quartz crystal, cut so that its mechanical vibration is resonant with the electric field, then mechanical oscillations are set up which can be communicated to a liquid and produce sound waves. Energies of up to 100 watts at one megacycle per second (mc), or even up to 30 mc with less energy, are possible, so that considerable power can be dissipated in the sound wave.

A consideration of ultrasonic action has been given by Fry, Wulff, Tucker, and Fry (1950). Although their work has not been concerned with viruses themselves, they point out the various physical factors which can enter into the process of sonic inactivation. It is clear that temperatures are developed which are quite able to account for many inactivation data.

Pressures Developed

The pressure, amplitude and density are related by the equation

$$P^2 = 2\rho VI \tag{7.1}$$

where P is the pressure in dynes per square centimeter, ρ is the density in grams per cubic centimeter, V is the velocity of sound in centimeters per second, and I is the intensity in ergs per square centimeter. For a case where 35 watts/cm^2 are developed, the pressure amplitude can be 10 atmospheres. Note that the pressure swings to low pressure also. Actually, as pres-

sures go, these are not large and will not normally be expected to have any great effect on a virus. The data of Johnson, Baylor, and Frazer (1948) quoted in Chapter 4 show that for tobacco mosaic virus the rate of inactivation at room temperature and 10 atmospheres pressure is not appreciable. It therefore seems likely that the pressure aspect of ultrasonics is not responsible for their effect on viruses.

Cavitation

Gas nuclei in a liquid can grow with great rapidity if pressure reduction occurs as in a sound wave. They also collapse with great rapidity, and this growth and collapse is called *cavitation*. If no dissolved gas is present in the liquid, cavitation cannot occur.

Temperatures associated with cavitation are not known accurately. By immersing powdered explosives which are not wet by liquids and then applying sonic energy, it is possible to measure the critical detonation energy. By relating this to the flash temperature of the explosive, the local temperature can be estimated. In a sound field of 20 watts/cm^2 at 1 mc, values up to 230° C were obtained.

These factors, and their biological action, are discussed by Harvey, Barnes, McElroy, Whiteley, Pease, and Cooper (1944).

The three possible physical agencies to watch in ultrasonic work are, therefore, *temperature*, *cavitation*, and *pressure variation*.

Sonic Irradiation Procedures

High-intensity sound in the upper audible range (10,000 cycles, or so) can be obtained with a magnetostriction generator. A metal which is magnetized changes length, and if a rapidly alternating current is fed from an oscillator into a coil of wire around a rod of steel, the alternations cause change in the length of the rod and these, in turn, can cause pressure changes in a liquid placed above the end of the rod. Since the permeability of the steel is high there is effectively an amplification, so that quite high powers can be fed into the liquid. Such sonic irradiation devices are available commercially. The design of an

irradiation apparatus is described fully by Krueger, Brown, and Scribner (1941).

True supersonic oscillations are produced by the electrical excitation of a quartz crystal, which changes thickness along a certain axis when an electric field is applied along that direction. The thickness of the crystal determines the frequency of oscillation, the thinner the crystal the higher the frequency, and, in practice, the only limit to the power available at frequencies below 1 mc lies in the power supply for the oscillator, which becomes cumbersome and expensive if power of the order of a kilowatt is needed.

The crystal and the associated electrodes are immersed in transformer oil. Above the crystal is placed a cell with thin upper and lower walls of any kind of plastic membrane, and the virus is suspended in solution in the cell. Power is then turned into the oscillator, and irradiation allowed to proceed intermittently (to control the temperature rise) for as long as is needed. The arrangements are briefly described by Newton (1951).

Sonic Effects on Viruses

An early observation of the effect of high-frequency sound on tobacco mosaic virus was reported by Takahashi and Christensen (1934) who subjected TMV to 9,000-cycle sonic action. They found that the recorded number of local lesions fell from 1,200 to 584 in 30 min, to 52 in 60 min, and to zero in 2 hr. The experiment was confirmed by Stanley (1934) who showed, however, that when the virus preparation was sealed in a vacuum and then irradiated almost no inactivation at all was found. It seems, therefore, that the inactivation is to be associated with cavitation.

To see how this can act, consider a small region of dissolved gas near the surface of the long, rod-shaped virus. Under high pressure, the gas is firmly part of a tight structure comprising the liquid and virus, with quite strong liquid-virus forces operating. As the pressure goes sharply negative, a gas cavity rapidly forms and forces the liquid apart. This can readily break the virus,

which will presumably break more readily at certain points. One possible study is, therefore, of virus length. This has been done by Oster (1947) and Newton (1951) and will shortly be described. In addition to this rather easily visualized process, the rapid development of a cavity will bend and distort the virus to some degree. This may easily cause it to become noninfective.

If the above picture of sonic action is accepted, it will be expected that viruses should vary greatly in susceptibility to sonic action. This is found to be so, and animal viruses seem to be much harder to inactivate than rod-shaped plant viruses. This variability in sonic action is shown in experiments of Anderson, Boggs, and Winters (1948). The relative loss of ability to form plaques after exposure to a magnetostriction oscillator in a water cooled cell is shown in Fig. 7.1. The seven T-series *E. coli* bacterial viruses were studied, and it can be seen that T-2, T-4, T-5, and T-6 lose their infectivity rapidly and in much the same way, whereas T-1, T-7, and T-3 are much less sensitive. T-3 appears to be inactivated in a nonlogarithmic way, whereas T-1 and T-7 follow a logarithmic relation. It is interesting that both T-3 and T-7 are very much alike in appearance in the electron microscope, being both spherical and about 450 Å in diameter, but are definitely different in sonic sensitivity. This was found to be true, although in a less definite way, for deuteron bombardment by Pollard and Forro (1951). A similar study of five *megaterium* phages was made by Friedman (1952) who also verified Anderson's work for T-1 and T-5 phages. Since a similar irradiation arrangement made by the same manufacturers was used, the results are comparable. They show logarithmic inactivation in all cases. The relation holding is, therefore, the familiar expression

$$\ln (n/n_0) = -k_s t$$

where n/n_0 is the survival ratio, k_s is a rate constant, and t is time. If we tabulate k_s from Anderson's and Friedman's data (using common measurements on T-1 and T-5 as mutual calibration), together with electron-micrograph dimensions, it can be seen that k_s is larger for the larger viruses. The rate constants

show a rough proportionality to the virus volume. This can be seen from Table 7.1. The existence, or not, of a tail seems to be unimportant, as T-7, with a short tail, is inactivated much like T-1, which has a long tail. More detailed studies are necessary before the nature of sonic action can be understood in detail.

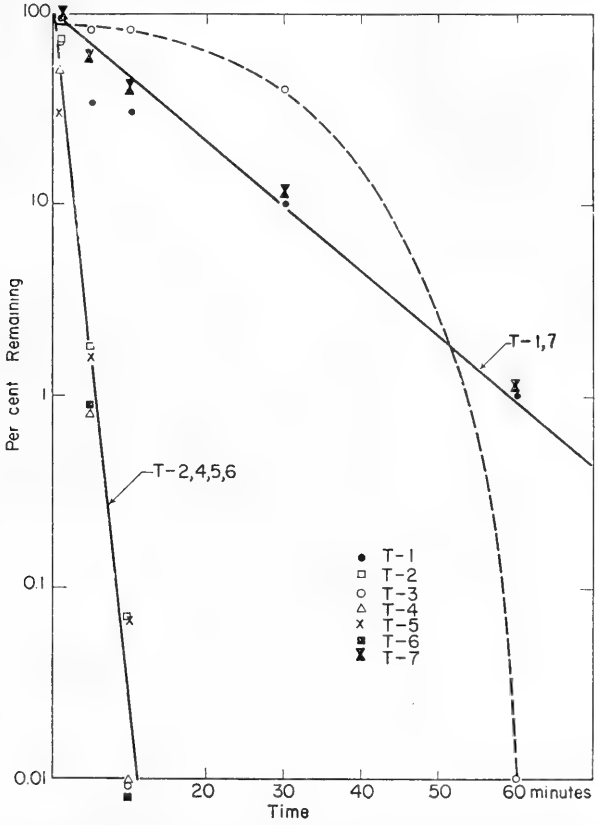

FIG. 7.1. Loss of plaque-forming ability of the T-series of bacterial viruses under sonic action, as measured by Anderson, Boggs, and Winters. It is clear that the viruses are not inactivated in the same way.

Three studies of sonic action on tobacco mosaic virus are of great interest. Two of these, by Oster (1947) and Newton (1951), are concerned with the effect of sound on the rod length of the virus particles, and the third, by Malkiel (1947), is concerned with the change in serological behavior after sonic irradiation.

TABLE 7.1

Virus	Rate constant, k_s (min^{-1})	Electron-microscope dimensions (Å)
T-1	0.24	500 head; 1,200 tail
M-1	0.59	650 " 2,900 "
T-5	1.0	1,000 " 2,000 "
T-2, 4, 6	1.0	700 " 1,000 "
M-4	1.6	1,000 " 1,850 "
M-5	2.0	760 " 3,000 "

In Oster's work, the virus was exposed to high-intensity sound at 9,000 cycles/sec. Under this treatment, the viscosity and stream birefringence of the virus preparation were both observed to diminish. As the sonic treatment was continued, the virus was observed in the electron microscope. It was clear that the average rod length had diminished and, in particular, that apparently rods broke into half sections, quarters, and eighths. Oster analyzes this process into a successive series and shows that at any particular fractional length there is an optimum time for which this length will predominate. Some of his data are shown in Fig. 7.2. It can be seen that sonic action affords a method of producing a change in rod length in a somewhat controlled way. From observing the ability of various sonically treated preparations to produce local lesions, Oster concludes that the infectious particle is 2,800 Å in length.

It is of interest that bringing the treated virus to the isoelectric point for three days at 37° C causes broken rods to aggregate. Oster found that these aggregated particles are not infective. They can be seen to be curved somewhat when observed on electron micrographs. These curved aggregates indicate, as Oster points out, that the length of 2,800 Å corresponds to that of the infectious particle.

Newton's studies were made with 7-mc sound produced by exciting a quartz crystal. The virus was placed in a Lucite irradiation cell immersed in transformer oil, and probably some internal heating occurred, although the outside was water cooled.

Infectivity was treated on bean plants at the two-leaf stage, and 10 repeats were taken for each determination. As in Oster's work, the nature of the virus was subsequently studied in the electron microscope.

It was found that after 200 sec exposure, the lesion counts were down to 5% of the original. Newton concludes that the ultrasonic irradiation of aged (and hence aggregated) virus suspen-

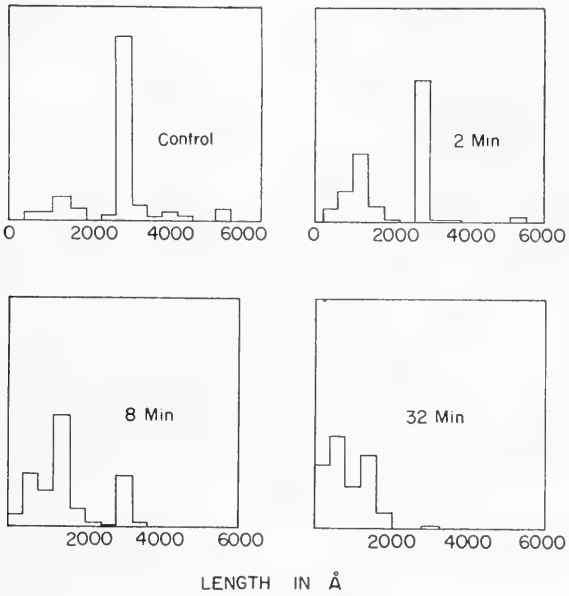

FIG. 7.2. Histograms of particle lengths of sonically treated TMV preparations, as measured by Oster. The appropriate infectivity is indicated and offers strong presumption that the particle length of 2,800 Å is the minimum effective,

sions produced more infectivity, due to dispersion of aggregation. This is an initial effect at relatively low power levels. The end-to-end aggregations become disaggregated into basic units 2,800 Å long with an increase in infectivity. At high energy levels, the 2,800-Å unit begins to fracture. First it goes at a constant distance from the end of the rod. The fragmentation becomes more and more random as the power increases, but it points to a weakness in structure at one definite point in the rod. This last is not quite what Oster found. The two frequencies of sonic ac-

tion are different in the two cases and may account for the different results.

The third investigation, that of Malkiel, concerns serological affinity after sonic action. Malkiel studied the precipitin reaction between virus and antisera. The antisera were of two kinds—antinormal TMV and antisonically inactivated TMV. The amount of antibody nitrogen precipitated was plotted as a func-

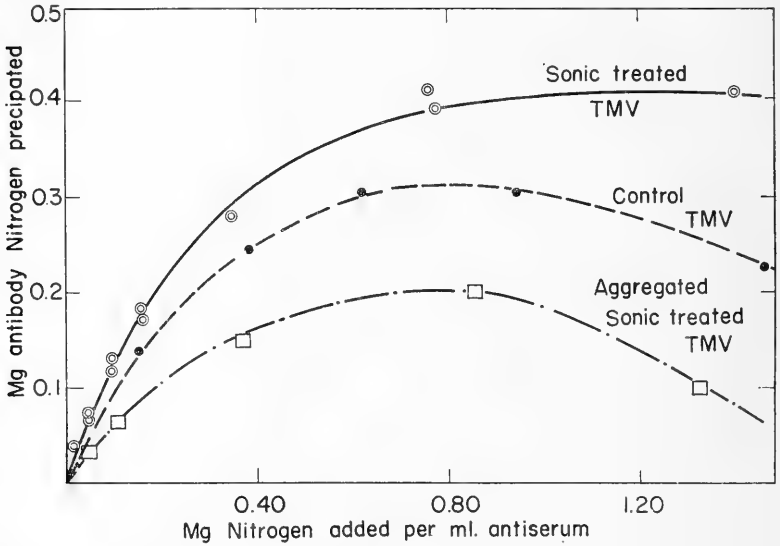

FIG. 7.3. Affinity between antiserum and TMV, as measured by Malkiel. The sonic treated TMV gives 30% more precipitate than does untreated, and much more than does reaggregated TMV.

tion of the amount of virus nitrogen added per milliliter antiserum with the results shown in Fig. 7.3. It can be seen that, for normal virus added to normal antiserum, the amount of precipitate increases, flattens, and falls in the usual way as the amount of virus is increased. When the sonic treated virus is used, the amount of precipitate reaches a high value, corresponding to a 30% increase over the normal virus. The average virus particle size was measured and was about half the predominant rod length in normal virus. The expected increase in surface area is about 7% for one-quarter-length rods. Thus the increase in area

alone is not sufficient to explain the results. Malkiel points out that there must be a repetitive antigenic unit throughout the virus which is probably quite small compared to the cross sectional area of the virus and which becomes exposed on breaking the virus. In addition, some steric hindrance to the attachment of antibody must be removed when the rods are shortened.

Antibody to sonic treated virus was also prepared by injecting rabbits, and it was found that more precipitate was formed with this also. This again argues for a simple repetitive unit.

When the aggregated virus, prepared by incubation at the isoelectric hydrogen-ion concentration, was used to combine with antiserum, less precipitate resulted. This is not unreasonable, as surface area becomes covered by aggregation, and the method of reassembly probably squanders more area at the joints than is needed by the normally attached virus.

This idea, of a small antigenic unit, is excellently confirmed by the deuteron bombardment studies described in Chapter 3 and lends some confidence to the inferential methods used in virus research.

Osmotic Effects on Viruses

Certain viruses will withstand suspension in a solution of high molarity. Under such circumstances the virus becomes permeated, in at least part of its internal structure, with high-concentration solute among its molecules of water of hydration. The osmotic pressure due to 4*M* NaCl is 90 atmospheres, and if a virus which has settled stably in such a solution is suddenly immersed in nearly solute-free water, the osmotic pressure will exert itself and an internal hydrostatic pressure will develop. In a large enough virus, the force so produced may cause a rupture at a weak internal point and so produce virus inactivation.

This effect was discovered by Anderson (1949). He found that T-2 phage was inactivated by the process of first immersing in 4*M* NaCl for a few minutes and then rapidly diluting the preparation with distilled water. He further showed that "ghosts," which had the tadpole shape of T-2 phage, were present and that they could absorb to the bacterium, although they were not in-

fective. The adsorption process includes both steps in attachment, the electrostatic and enzymatic, for the ghosts can cause bacterial cell lysis from without.

Further study of this effect was made by Herriott (1951) who showed that osmotic shock released the desoxyribose nucleic acid (DNA) from the virus and left behind ghosts which were free of nucleic acid. These ghosts still performed the attachment and lytic functions. He made clear that this affords a powerful method of separating the infective and surface functions of a virus.

This suggestion has been followed up with most interesting results by Hershey and Chase (1952). The basic technique in this important series of experiments is the use of P^{32} and S^{35} to trace separately the nucleic acid and protein of the virus. The phosphorus content of T-2 phage is 96% in nucleic acid (Herriott and Barlow, 1952). There is no sulfur at all in nucleic acid, but some in protein, although, actually, proteins containing sulfur are not typical. Thus the fate of P^{32} tells the fate of nucleic acid in any process, and S^{35} reveals the course of protein.

In some preliminary experiments, Hershey and Chase show that the findings of Anderson and Herriott are confirmed and that, in addition, the osmotically released ghosts contain all the serological affinity of the phage, and that they are adsorbed to susceptible bacteria and not to any bacteria. They then followed up a discovery to Graham (see Hershey and Chase, 1952, p. 43) that the adsorption of T-2 to heat-killed bacteria permitted enzymatic breaking up of the DNA by a specific enzyme (DNAase) which was powerless in the unadsorbed phage. Using radioactively labeled phage particles they showed that after phage was adsorbed to heat-killed bacteria, the action of DNAase released 76% P^{32} into solution, compared to 13% released in the absence of enzyme. Adsorption to live bacteria does not free the DNA to enzymatic attack, for only 8% P^{32} was rendered soluble after phage adsorption to live bacteria and treatment with enzyme. On the other hand, heating bacteria after infection produced a release of P^{32} nearly equal to that from heat-killed bacteria.

Freezing and thawing of infected bacteria also rendered the DNA vulnerable.

These experiments, therefore, strongly suggest that there is a protective coating of protein around the nucleic acid, which is broken by absorption to nonfunctioning bacteria but is supplanted by some other cellular protection when the bacteria are metabolizing.

To check this, Hershey and Chase studied the fate of S^{35}. They showed that all the S^{35} is contained in the osmotic ghosts and is in the antigenic part of the virus. They further showed that, after virus attachment, violent agitation of the infected bacteria releases 75% of the S^{35} and only 15% of the P^{32}, while still leaving some agent inside the bacterium capable of multiplication of further phage particles. In any event, apart from such treatment, it is found that less than 1% S^{35} is found in the next generation of phage, in contrast to 30% P^{32}.

The following remarkable picture of the nature of T-2 phage emerges. The dry virus is approximately 20% nucleic acid and 80% protein by volume. According to Anderson, attachment is by the tail, which can be seen to be wide and flat at the end. This occurs by the dual method already described, and thereafter the nucleic acid must in some way be pulled inside the bacterium, possibly by a simple pressure difference characteristic of the difference in physical state of the bacterium and virus. The force due to a tendency of the surface to contract would be sufficient for this purpose. The protein coating, with the entire antigenic surface, is then left behind and can be shaken off without particularly impairing the ability of the specific phage DNA to perform its function inside the bacterium.

The resulting picture of T-2 phage is shown schematically in Fig. 7.4. The nucleic acid is shown as a long, thin structure contained both in the tail and in the head. This is attached to the protein by presumably weak forces, but sufficient in strength to hold the surface intact until contact with the bacterium is made. In this figure, no attempt has been made to distinguish between sulfur-containing protein and other, although it is only

the former which has been traced by Hershey and Chase. Probably the inner protein layers are more tightly bound to the nucleic acid and go with it into the bacterium. Strong, specifically distributed charges must exist at the point of attachment, and

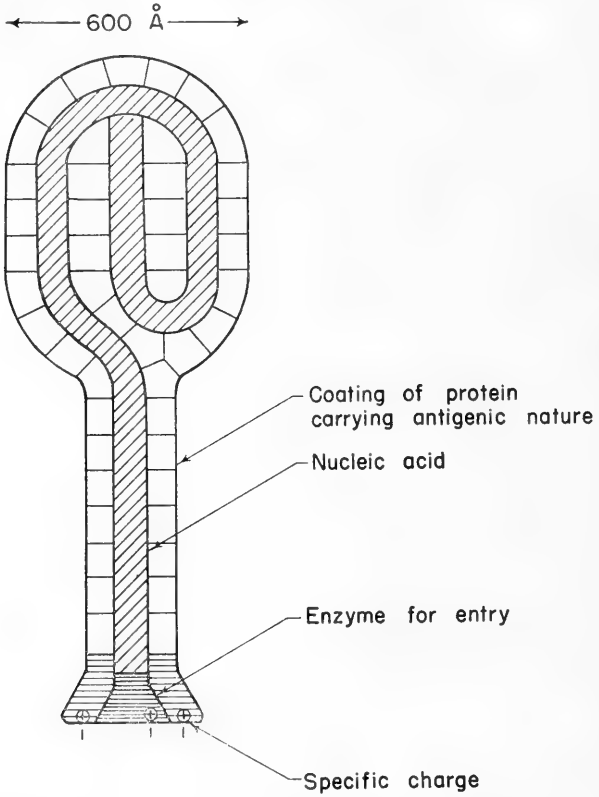

FIG. 7.4. Schematic drawing of T-2 phage based on osmotic shock and radioactive studies. The phage attaches by the tail, enzymatically digests an opening in the bacterium, releases its nucleic acid, and leaves the protein coat outside.

the enzyme which takes over after the electrical attachment phase must also be located near there.

Some very interesting thoughts follow from this picture. If attachment is at the tail, as this picture suggests, then the fact that attachment occurs at every collision, as shown by Schles-

inger and Delbrück (Chapter 5), must be explained. Although a collision of so large an object is a slow and gentle matter, nevertheless, there must be an *absence* of specific charges except at the tail, or such attachment would hold the phage in the wrong position. If the only charges are on the tail then they must (*a*) be specific and (*b*) exert enough force to orient the whole phage as it approaches the bacterium. For the second purpose, we can suppose that force must extend over several hundred Ångstrom units, and this can only be due to unneutralized opposite charges. An energy equal to that of thermal agitation cannot be provided by single, opposite charges, if the local dielectric constant is taken as 80, but needs 10 or more to be effective at 100 Å. The mutual repulsive force, if these are all on the virus in the confined region of the tail, is so great that it might well interfere with nucleic-acid-protein binding. So the charge must be primarily on the bacterium. Now, for the first purpose, there must be a specific, related configuration, otherwise nonspecific binding would take place. We are thus led to the idea of three or four singly charged groups, each well away from the others, on the end of the tail of the virus. The charge specificity thus becomes a property of the organization of whole protein molecules between one another and not of the surface of each separate molecule.

A second important point is made by Hershey. It will be recalled that both for TMV and SBMV there is good evidence that surface antigens are relatively small, probably only a fraction of a protein molecule. Bearing this in mind one can argue thus. Since, (*a*) the sulfur antigens are capable of being left behind at attachment, (*b*) they are the same in the progeny resulting from the same attachment, and (*c*) they are different from any bacterial antigens, there must be a way in which the nucleic acid can coerce a *small, detailed rearrangement* on many protein molecules or their precursors inside the bacterium. In this way, new, identical antigen molecules are made in a few minutes. This inescapable fact throws a strong light on the thoroughness with which bacterial metabolism must become virus metabolism after infection. One's respect for the power of a biologically intact nucleic-acid structure rises to even higher levels.

The lines of work described in this chapter are capable of much elaboration. Comparable pictures for other viruses would be of the greatest interest and should be forthcoming before long.

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CHAPTER EIGHT

VIRUS GENETICS, VIRUS MULTIPLICATION, AND VIRUS PHYSICS

Stripped of their practical interest as agents causing disease, the deep reason for studying viruses is to find out how they work, particularly because they may be representative of how living systems function in their very primary processes. The physicist has an even more absorbing interest, which is to find out if physical laws are sufficient to explain all virus processes. It seems reasonably certain that viruses are not concerned with the structure of the atomic nucleus, nor with relativistic electrodynamics, nor cosmology. These are the great uncertainties of physical theory today. Instead, the simple electrical laws and laws of quantum mechanics, which have proved completely adequate to explain the findings of chemistry, and which appear to be on a solid foundation, should serve to explain virus behavior. These laws cannot be applied as yet, because neither the structure of viruses nor of their environment is yet sufficiently accurately described. Nevertheless, the biophysicist cannot help looking with some anxiety, as the description of viruses and their behavior becomes more and more definite, to see whether the parts and their functions are of the right type to be susceptible to physical description without invoking new principles. He remembers that the nature of the spectrum of black-body radiation, known toward the end of the nineteenth century, carried in it the downfall of classical physics, then moving so confidently forward. Perhaps there is already a clear conflict between virus behavior and physical laws—a conflict which requires deeper insight to appreciate than we have yet brought to bear. Perhaps, on the other hand, a resolute confidence in physical laws can even now be

used by an astute theorist to predict undiscovered properties of viruses.

We have already seen that certain aspects of virus action, most notably the first stage of attachment to the host cell, are very readily described (though so far no exact theory has been developed) in terms of electrostatic attraction. There seems to be no reason to believe that any enzymatic processes which follow are in any way demanding of new principles, because enzyme action in general seems to conform to basic physico-chemical principles. However, neither of these can be more than rather rudimentary aspects of virus behavior. At the time of writing, the most penetrating studies of viruses are concerned with the phenomenon of virus recombination and the genetic studies which have ensued. Therefore, even though it takes us beyond the confines of physical studies, we describe here some of the genetic findings, because these must be part of an over-all physical description of virus action when (and if) it is one day complete.

Virus Recombination and Virus Genetics

Viruses which are essentially similar to one another can, in some instances, be distinguished by characteristic behavior. For example, of two viruses, one can have a short latent period and a large burst size and so can produce lysis at a rapid rate. The plaques so formed are then distinguishable from another quite similar virus which lyses more slowly and produces smaller plaques. Or again, two closely related bacterial hosts may act as indicator for one but not the other.

In 1946, Delbrück and Bailey made the important observation that if two phages of differentiable type infected a host cell, then the resulting burst contained new types of phage differing from the originals, and which retained their behavior after many future generations. This process of recombination has given rise, largely owing to the work of Hershey, to a whole developed subject of bacteriophage genetics which is of the greatest interest.

Such recombination has also been observed in influenza virus by Burnet and Lind (1951). They have shown that three strains:

NWS with no enzymatic or indicator activity, but neurotropic; WSE with firm noneluting adsorption to red cells, and WSM, a very heat-resistant hemagglutinin, can recombine after multiple infection to produce recombinant variants in much the same way as found by Hershey and Rotman (1949). Thus, recombination can be considered as a property of animal viruses also.

Stated very boldly, the way the geneticist works is by discovering a means of genetic combination, for example mating, and then analyzing the progeny in terms of some clear property to see what proportions of the progeny show the property. "One white, one black and two khaki" is fundamentally what is sought, although the whole beautiful and intricate pattern of modern genetics contains the description of genotypes involving thousands of separate genes. Phage genetics has only a small number of phenotypic expressions to consider at the present moment, but is rendered a powerful and challenging subject by the ability to observe large numbers of progeny and the fact that triparental recombination is also observed, thus adding another dimension to the range of possible experimentation.

To illustrate the method of study, consider multiple infection of *E. coli* cells with T-2 phage. These can be different strains of T-2, for example T-2*r*, a rapidly lysing form, and T-2*h*, a phage which will multiply in *B*/2, a different form of the host bacterium. It is possible, by plating on a mixture of *B* and *B*/2, to tell the proportions of *r* and *h* and of recombinants between them. The rapid lysis feature can be exhibited by 15 different types of virus corresponding to 15 mutant forms. These are numbered r_1 to r_{15}. Now, if r_1 and *h* are used to produce a mixed infection, a process represented by $r_1 \times h$, there result, from *many* bacteria, the following proportions: 30% r_1, 30% *h*, 20% r_1h, and 20% with neither feature, the wild type. These figures are not the standard proportions of genetics, but this is not a standard process. The important fact is that relatively high proportions of the recombinants r_1h and the wild type are produced. If, in place of a mixed infection of *h* and r_1, *h* and r_{13} had been used, a much smaller recombinant proportion, only 0.8%, is found. Hershey infers that *h* and r_{13} are closely linked, so that they go together

very commonly. In common genetic terms, they are on the same linkage group and close together.

To explain their results, Hershey and Rotman (1949) use three linkage groups and 17 total genes.

In all of the above, only biparental recombination has been considered. If, now, infection is carried out with three phages differing in three genetic loci, a much more complicated pattern results. Delbrück (1951) has indicated how this may be analyzed, and has applied his treatment to some of the simpler experimental trials. The remarkable result emerges that the theoretical analysis stands up in the face of experimental findings, but that four rounds of mating between genetically completed viruses must take place in the time of a single burst. This means four rounds in a few minutes, since it certainly takes a major part of the latent period to assemble genetically finished phage. This is very rapid and is surprising, but at present has to be accepted as in the nature of virus genetics.

The two major conclusions to be drawn from virus genetics are, therefore, that there are many genes per virus, possibly 20 to 100 in number, and that viruses which are complete as regards their genes can "mate" inside the bacterium and do so very rapidly. A secondary conclusion is that some of the genes are linked, or at any event combine with one another with low probability.

Before trying to see what this entails in terms of physical structure, we consider a second important and recent line of work on phage multiplication.

Bacterial Virus Multiplication

The simplest physical idea of virus multiplication is the *template* idea. According to this, the entering virus, or a part of it, is a template which, acting as a firm structure in the midst of the metabolic turmoil of the bacterium, causes copies of itself to be formed by what amounts to a printing press operation. Although all features of the idea are not simple, the notion that short-range physical forces compel the aggregation of like particles around the template, and hence produce a replica, is not unattractive.

To test this, two experiments have been carried out and are here described.

Radiophosphorus Studies of Phage

If bacteria are grown in a medium containing P^{32} or S^{35}, and then infected with bacteriophage, the resulting bursts will contain phage which carry the radioactivity to some extent. These labeled phage particles can then be used for various kinds of studies of virus processes. We are here concerned with the question of whether a virus particle acts as a template and stamps out other particles, or operates in some other way, for example, by growth and division. Some evidence, but not final evidence, can be obtained from following the course of radioactivity in the second generation of virus.

If radioactive phage is used to infect a bacterium, the question arises as to how much of the radioactivity is associated with a hypothetical template, and how much with a part which is not essential to the reproduction process. The radioactivity in the latter part will presumably interchange by chemical processes with the bacterial cytoplasm and so be removed from the phage. This is indeed found to be so. If the total phage released in the first generation is harvested and separated by centrifugation from the bacterial debris, it is found to contain 30% of the P^{32} originally used in the infecting phage (Kozloff and Putnam, 1950). Now it may be presumed that *this* P^{32}, which has had all the nongenetic phosphorus removed from it in the first generation, will remain attached to the genetic part and so, if all the phage is again harvested in the second generation, the same total P^{32} should be measured. Actually, for T-4, Maaloe and Watson (1951) report that only about 30% is again recovered. Some preliminary experiments by Forro for T-2 showed the same kind of result. In view of this, it seems hard to believe that an indestructible template, in the physical sense, is part of the multiplication process. It is also remarkable that as high a turnover as is observed takes place. It would be expected that growth and division might spread the P^{32} among many more virus particles, but one would think that a retention of a high percentage should occur.

This indicative experiment has been followed by a tedious but powerful study by Luria (1951). Like other mutations, bacterial virus mutations take place spontaneously. This is related to the rearrangement of a molecular order in consequence of the thermal agitation of a large molecule. Whatever the cause of such mutations, they occur, and they do so while the phage is active, namely during the infective time in the host. Now the presence of a mutation can be detected by the ability to produce lysis in a host which is resistant to the parent strain, and so produces plaques. The use of mixed bacterial cultures, B and $B/2$, where $B/2$ is resistant to T-2 but not T-2h, is a convenient device, for, unless both T-2 and T-2h are present, a cloudy plaque, containing developing $B/2$, is formed. The rapid lysis mutant T-2r can also be recognized by the larger plaques formed.

Luria's experiment consisted in patiently observing the frequency of occurrence of two mutants, T-2r and T-2w, in 23,000 bursts of bacteria infected with a strain T-2L. In each burst, the number of mutants was observed, and the fact that in a fair number of cases 10 or more mutants per burst occurred was recorded. In these cases, the identity of each mutant was checked by using them in mixed infections and showing that no new recombinant forms resulted. To see what the experiment can tell us, consider three possible ways in which these mutations can occur.

The first, and most obvious, is that any phage, independently of reproduction, can mutate at any time with a definite probability. This is a rather odd idea since it supposes that the mutant phage produces no mutant progeny. It could happen if a master template phage were stamping out replicas and some of the replicas subsequently underwent a molecular rearrangement which was capable of making different phage when the changed replica had its turn in a new bacterium. If the average of all mutants turned out to be x per burst, then the Poisson formula should hold, and the number of times that n mutants would occur would be determined by $P(n)$, where

$$P(n) = \frac{e^{-x}x^n}{n!}$$

The characteristic behavior of $P(n)$ is that if small values of n are to be expected on the whole, then values several times larger have a very low probability. Thus, in Luria's work, the average number of mutations per plate (several bursts to a plate) was 0.25. We then have $P(1) = 0.195$; $P(2) = 0.024$; and $P(3) = 0.0022$. Thus there is roughly a factor of 10 less for each

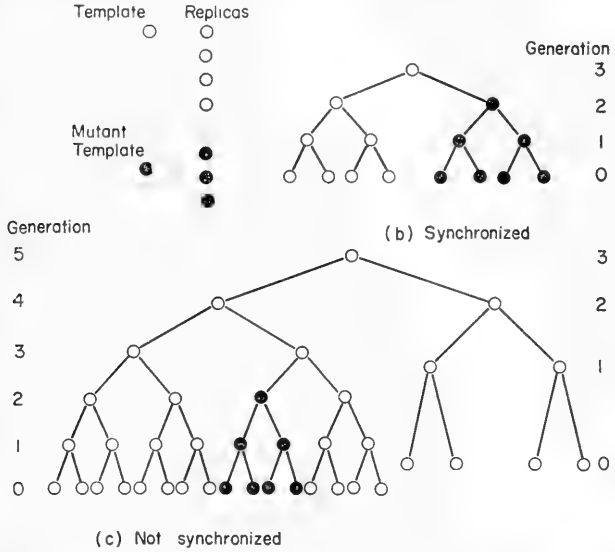


FIG. 8.1. Three different methods of observing spontaneous mutants according to three processes of multiplication. (a) is the template which mutates. (b) shows a synchronized exponential multiplication, and (c) a non-synchronous process. Illustrates Luria's experiments on mutation of bacterial viruses.

increase of one in the number of mutants per plate. This very definite characteristic should be easy to confirm.

A second possibility is essentially the same but it supposes that mutations take place in the template itself which thus, after stamping out a certain number of replicas, changes its character and stamps out a whole new succession of mutant phages. The character change can occur at any time, with no preference, and so this process should cause a distribution of mutants per burst which extends evenly from one to the burst size. This process is

illustrated in Fig. 8.1, where the changed virus is shown as shaded. Since the mutation point is not specially selected, any number of mutants is as likely as any other.

The third possibility is that reproduction by some kind of growth and division takes place. In principle, division into n units is possible; Luria considers division into two, as indicated in Figs. 8.1b and 8.1c. The simplest method is that shown in Fig. 8.1b, where each generation of the progeny occurs at the same time. It is more likely that the actual process is the same, but is not synchronized, and this is shown in Fig. 8.1c. The important point is that exponential growth follows each mutation, so that the progeny of the mutant, which also consists of mutants, develop as shown.

To compute the number of mutants expected we can argue thus. Designate the generation 0, 1, 2 . . . as indicated. Then a mutation at the kth generation produces 2^k mutant phages. Call this x. The total number of phage particles present at the kth generation is $N/2^k$, where N is the total number present at the end, the zeroth generation. If the chance of mutation per particle at (or between) each generation is m, then the number of mutants at the kth generation is $mN/2^k$. This is mN/x. Thus the number of groups (clones) of mutants containing x particles per group is inversely proportional to x.

This assumes synchronism. If the generations are not synchronized, then any particular value of x can be derived from a value of k occurring at a stage where division has been slow and fewer individuals have been present, or it can correspond to a well populated generation. These variable factors do not alter the essential form of the distribution in which the proportion of clones of size x, or greater, should be inversely as x.

Luria's data are tabulated below. Two mutants were closely observed. The figures are connected for the small number of cases where the original infecting particle is itself a mutant.

It can be seen that the proportion of clones with high values of x is not extremely small, as required by the Poisson distribution for random mutation. This is thus experimentally ruled out. Also, the proportion of clones *does* become less as x increases,

TABLE 8.1

Number of mutants, x, in a group or clone	Number, y, observed with x or more (r mutant)	yx (r)	Number observed with x or more (w mutant)	yx (w)
1	85	85	98	98
2	38	76	52	104
3	29	87	34	102
4	18	72	23	92
5	16	80	19	95
6	16	96	18	108
7	14	98	11	77
8	13	104	9	72
9	12	108	8	72

instead of being uniform as required for the mutating template method. So that again this is experimentally disallowed. On the other hand, the requirement imposed by the exponential growth method, that the number of clones with x or more mutants vary as $1/x$, is quite reasonably well fulfilled. Perhaps more work needs to be done before strong assertions are made but, within the compass of the data of this very well conceived experiment, the growth method of Fig. 8.1b or 8.1c is definitely indicated.

Since the next generation is produced by the splitting of one individual into two individuals, there is really no way in which "genetic" P^{32} can be fixed in one generation and held there, because the P^{32} will be divided and will then take part in all the characteristics of the new phage generation, not all of which need be "genetic." So the experiment of Kozloff and Putnam, Maaloe and Watson, and Forro is in keeping with Luria's findings.

Summary of Pertinent Facts About Viruses

In order to guide the formulation of hypotheses about virus multiplication, we give here a summary of facts about viruses which seem significant. Many of these facts are derived for bacterial viruses, but evidence is growing that animal viruses conform to the same pattern. Plant virus may also do so, but entry into the infected cell is probably by different means. The significant points are:

(*1*). They are always nucleoprotein.

(*2*). They are roughly 25% nucleic acid and 75% protein, with wide variations possible.

(*3*). They attach electrostatically to the host in the case of bacterial viruses. Co-factors may be needed.

(*4*). They enzymatically confirm their entry into the bacterium.

(*5*). Not all the protein enters, the sulfur-containing part remains outside.

(*6*). Bacterial metabolism is rapidly shifted to viral metabolism after entry.

(*7*). The timetable for T-1 is such that 300 new units are formed in 13 min.

(*8*). About 1,000 surface specific antigens exist.

(*9*). Polypeptide absorbed ultraviolet light is *less* effective in causing virus inactivation. Nucleic-acid absorbed light has the maximal effect.

(*10*). Damage by heat, ultraviolet light, and X-ray or deuteron bombardment can lengthen the latent period.

(*11*). Multiple infection by ultraviolet inactivated virus particles can cause reconstitution and growth of virus.

(*12*). Ultraviolet inactivated virus becomes reactivated to some extent by visible light while in the bacterium.

(*13*). About 50 units are responsible for shortening the latent period.

(*14*). Genetically complete virus particles are formed and multiply by something akin to division.

(*15*). Genetically complete virus particles can exchange genetic units very rapidly among several particles.

(*16*). Mutants which recombine have the same serological behavior.

(*17*). No cross reaction between phage antisera and bacterial antisera exists.

Description of Present Knowledge of Virus Multiplication

Putting together a story from the summary just given we can see it as follows. The virus is a completely inert, "physical"

object in between its life inside the host bacterium. It carries, in solution, charged groups which can attach and bind ions to them and so present a specific charged grouping all over its surface in some cases, and at definite spots in other cases. When the right number of ions are bound, the virus can be attracted to the oppositely charged (also specifically grouped) bacterium and there be held by electrostatic forces. While in this condition it can be eluted from the bacterium by changing the ionic strength to one which is not suitable, and agitating strongly. If the temperature is right, this kind of attachment is superseded by an enzymatic action which makes a sufficiently large opening for the long and thin nucleoprotein of a virus to enter, while leaving outside a purely protein part which is not concerned with reproduction.

The organization of the nucleoprotein which has entered is now reduced to a lesser degree, and the process of active multiplication begins. Because the latent period can be lengthened by damage which does not prevent multiplication, there are presumably nongenetic units present on the entering nucleoprotein. These are quite possibly a group of similar enzyme molecules or enzyme precursors which disperse through the bacterium and start the almost instantaneous change in its metabolic process.

The genetic part of the virus consists of several units of nucleic acid, or nucleoprotein. It is multiple, and some genetic factors are relatively tightly bound together whereas others are readily separable. Before ultimate virus assembly, these units are capable of exchange between individuals. The exchange takes place rapidly and very thoroughly, as four rounds of "mating" can take place in a few minutes.

Virus construction is concerned with near neighbors, for the exponential growth process requires something like the construction of a new virus, either by growth and division of the old virus, or by the growth of a new one very close indeed to it, certainly close enough to be within the range of some type of molecular forces.

While this takes place, a steady change of the protein character in the bacterium goes on and some 300,000 specific molecular protein units are produced, ready to form the outer protein coat-

ing around the nucleic acid or nucleoprotein internal structure. Although these protein molecules carry the enzymes which cause cell lysis among their number they do not cause lysis at once, for many nearly complete individuals exist inside the bacterium a minute or two before lysis occurs.

Among the cell debris after lysis are units which can combine specifically with antiphage so that exact assembly of all possible virus components is apparently not a necessity, a rather welcome lapse in an almost unbelievably efficient and coordinated process.

The taking over of metabolism mentioned several times before must include detailed molecular rearrangement, both on account of the small size of serological unit which is remade in quantity and because the bases present in virus nucleic acid have different proportions from those in the bacterium.

The 300 or so phage particles now enter a resting phase unless more specifically suitable bacteria are present, in which case the process goes on until all bacteria are lysed.

This brief description is intended to be suitable for this book. More can be learned from Luria's article in *Viruses 1950*. The question now arises as to what kind of physico-chemical processes underlie this amazing biological feat.

Description of a Virus

From time to time in the previous pages we have drawn pictures of viruses in terms of certain properties shown by the particular experimentation which we had just described. Each of these pictures was useful in clarifying the conclusions gained about virus structure, but none of them expressed in any detail the known facts about viruses.

We have detailed studies on two or three plant viruses and several bacterial viruses, notably members of the selected T-series of *E. coli* phages. In Figs. 8.2 and 8.3 we show pictures which represent educated guesses at the structure of two plant viruses, *southern bean mosaic virus*, and *tobacco mosaic virus*. In Fig. 8.4 we show a bacterial virus which is *T-1 phage* with a dash of the properties of T-2 to fill out the incomplete studies on T-1.

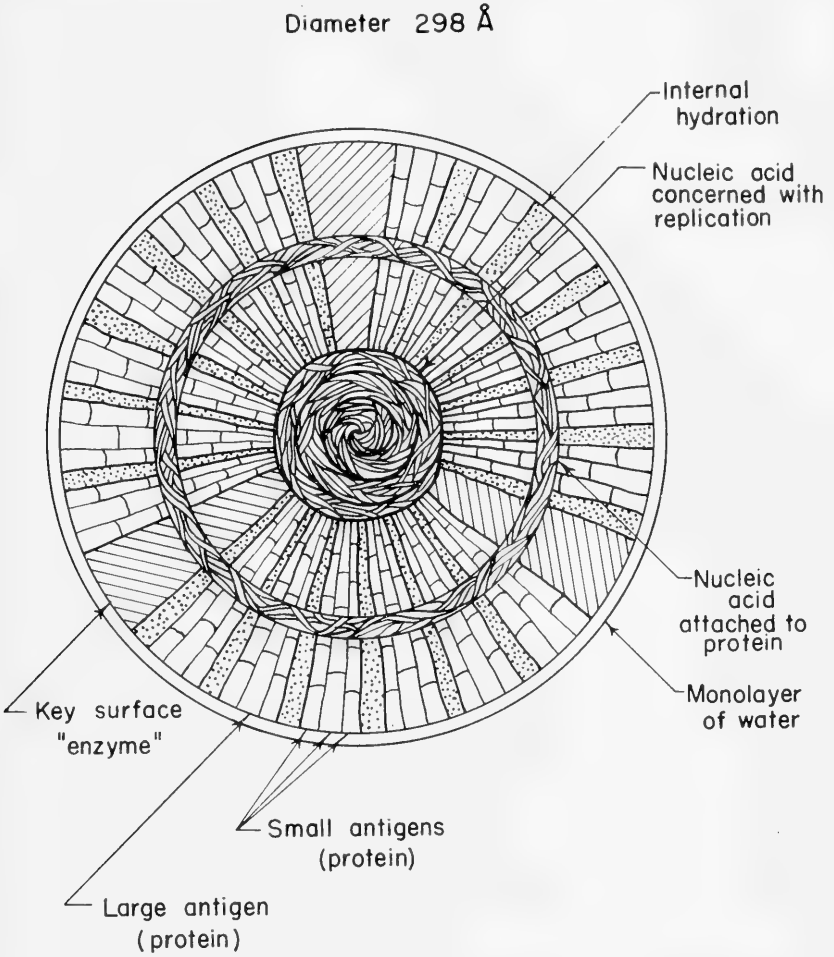


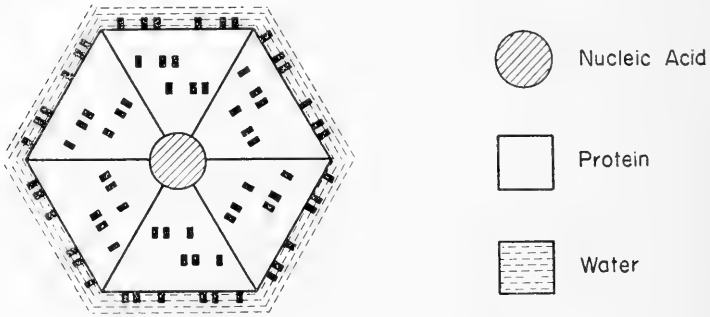
FIG. 8.2. Representation of southern bean mosaic virus in a manner which incorporates some definite and some speculative knowledge. The location of the nucleic acid is quite speculative.

In Fig. 8.5 a very incomplete presentation of an animal virus, Newcastle disease, is given.

Taking southern bean mosaic virus first, the most remarkable fact is the accurate spherical shape. Second is the existence of a double repeated antigenic pattern on the surface. This plant virus forms crystals, and seemingly also does so inside the in-

fected plant cells. The nucleic acid is ribose nucleic acid and it is bound relatively tightly to protein, although this is not characteristic of all plant viruses, and TMV is an exception. It is considerably radiation sensitive, and so is probably very simple and functional in structure. It has a high proportion of internal water of hydration.

In the representation, the nucleic acid has been divided rather arbitrarily into two parts, one at the center which is supposedly primarily involved in the self-duplication process, and one



TMV

FIG. 8.3. End view of tobacco mosaic virus. Again, the location of the nucleic acid is speculative. The repeated small antigenic pattern is shown, with oriented, aromatic amino acids forming a part of each antigen.

attached firmly on both sides to protein and having the antigenic characteristic under its control in consequence. It has also been assumed that a relatively small number of surface molecules are concerned with enzymatic digestion of the surface of the plant cell. These are shown separately. The two antigens are indicated by making the small one a substructure in the large.

In the next figure, the end view of tobacco mosaic virus is shown. The long hexagonal rod shape has already been shown in Fig. 2.13, so only the end is shown here. There are really only two features which need comment. The first is the fact that 6% of the virus is nucleic acid, which rather readily separates from

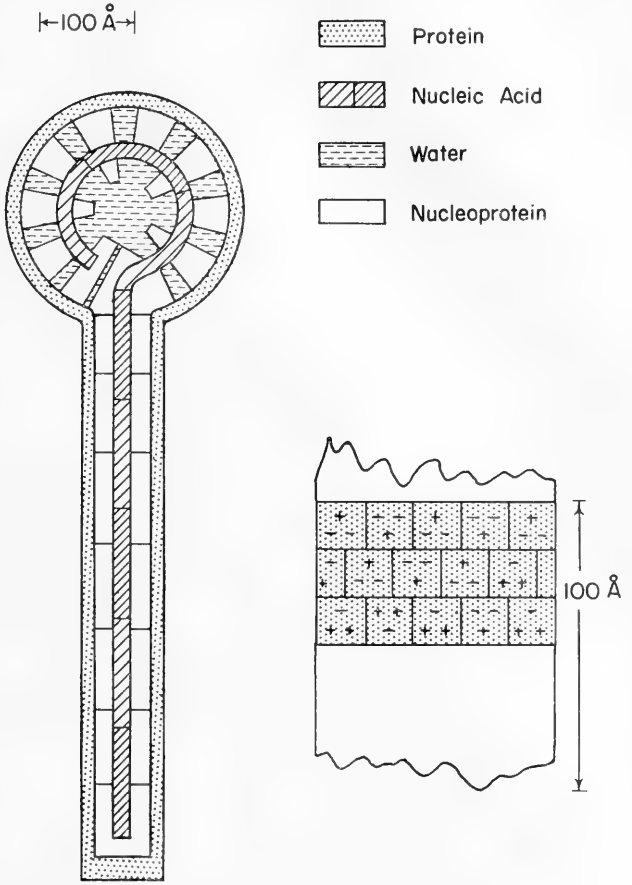


FIG. 8.4. Suggested structure for T-1 bacteriophage. The hook shaped central part is the nucleic acid essential for multiplication. If one-third of this is intact, the virus will kill bacteria. About 50 enzyme-like molecules are required to produce a short latent period; these are indicated as nucleoprotein. The whole is surrounded by a protein sheath, unessential for operation in the bacterium. An enlarged view of the antigenic part, with surface charges for attachment, is shown.

the protein. To indicate this in a token way, it has been placed as a long rod in the center of the virus, with protein units attached to it. Obviously, other ways exist, but it seems plausible that the nucleic acid extends down the virus. The second point concerns the antigenic units, which are small. In the figure, they

have been represented as a pattern of aromatic amino acids, which Butenandt has shown to be oriented in the virus. The pattern continues throughout the whole volume, and so has been

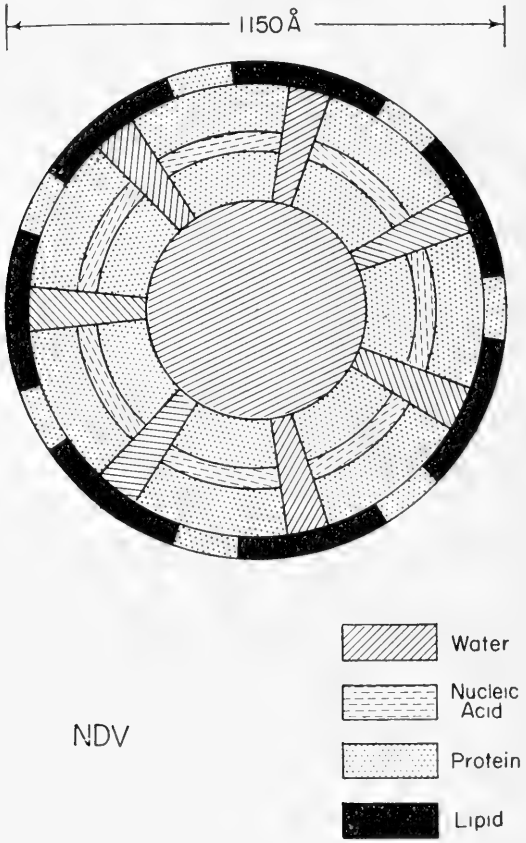


FIG. 8.5. Newcastle disease virus. This has an outer layer of lipid, through which project the hemagglutinins. The structure is simple and must be capable of unrolling in the infectious state. It is probably heavily hydrated.

shown on the end. A small layer of hydration has been indicated, perhaps rather overconfidently, as bound on the surface.

The third virus pictured is T-1 bacteriophage. Radiation studies show this to have a complex morphology, and an attempt to represent it schematically has been made. The nucleic acid is shown as made up of eight units, all attached to protein. The

ultimately highly vulnerable part of the virus is this, and unless it is damaged the virus will survive, although with impaired properties. The eight units are thought of as eight genetic components. They can mutate and, if such forms are stable, recombination could occur. The outer layer of protein, which does not enter the bacterium, is shown as an intact sheath. The protein units which control the latent period are shown as white blocks. The tail of the virus (the most convenient part to draw) is shown in more detail and is broken into a mosaic of antigenic units carrying a pattern of charge distribution which is specifically related to the charge distribution on the bacterium. Undoubtedly the virus is hydrated inside, and this is shown. The outer hydration has been omitted, as its thickness is a pure guess.

Finally, on very slender evidence, we show Newcastle disease virus. This is guessed at as spherical in the resting state, but is probably formed by coiling up an elongated structure which is the form it takes in the host. The lipid fraction is shown on the outside, with islands of protein penetrating through to form the hemagglutinins, which are of about the size indicated by deuteron bombardment. The virus is certainly very much hydrated, and this has been indicated as partly in the center and partly extending between the nucleoprotein units. The virus is a poor antigen, probably due to the lipid layer. It is very radiation sensitive, almost as much so as TMV, which argues for a rather simple internal structure.

We feel like insisting that these pictures be treated as speculative and tentative. However, in the author's experience, they have excited much interest and, after all, presumably viruses do have a functional internal structure—so why not say so. It is only in the event that these pictures should be treated as authoritative descriptions of the viruses that harm could be done. Probably in five years such authority can be justified—but not at the present time.

The Energy Turnover in the Host

One basic process which must be concerned with virus multiplication is the energy turnover, or metabolism, of the host. This

is taking place rapidly, as in all active living systems, and the nature of the process is of deep interest. In many of the systems with which physics and chemistry have to deal, the transfer of energy requires the generation of a certain amount of random thermal motion and its transfer partly to different random motion (at a lower temperature) and to definite linear motion. This is inherently impossible in biological systems which are at one uniform temperature. Schrödinger (1945) has pointed out the general mechanism by which this biological energy transfer takes place, and Delbrück (1946) has shown very clearly how it operates in one or two special cases.

Energy turnover takes place by means of atomic rearrangements. These do not take part in a statistical system, they have no "temperature," but rather involve a direct and very efficient method of moving energy from one form to another. Thus a loosely bound phosphate group can become a tightly bound group with the release of 2 or 3 ev of energy. However, the released energy does not have to cause random thermal agitation, but can produce the necessary excitation to break a bond and permit a new configuration which carries a high percentage of the original released energy. Almost certainly the function of enzymes is to hold systems in place so that such energy transfer can take place through the enzyme molecule and effect the required change. The energy shift is rapid and at high efficiency.

The host cell has thus an equipment comprising specialized enzyme molecules, small co-factor molecules which can form temporary structural unions, as well as a basic medium of small molecules and ions. A part of the necessary behavior of the cell is the assembly of nucleic acid and protein structural units. These are formed from the simple molecules of the medium supplying the host, either directly after assimilation through a membrane as in the case of a bacterium, or through the more complex processes of an animal or plant. These units evidently carry with them a specific character, probably imposed by the general nature of the enzymatic protein, which is a simple repetition of a rather limited number of amino-acid side chains in the case of a small polypeptide precursor, or an order to the nucleosides in the

case of a nucleic-acid precursor. It is this intimate specific character which is expressed in the antigenic nature of the bacterial contents.

In the bacterium, use is made of the nucleoprotein precursors either as enzymes for causing the synthetic metabolism to proceed rapidly and effectively, or as units which form part of the new genetic material necessary to carry the basic cellular character over into the next division.

None of this process is clearly understood at the present time. It seems speculatively reasonable, however, that rather rapid chemical rearrangements can be made, utilizing energy by direct transfer to form either nucleic-acid units of molecular weight 2,000 or so, or small proteins of the same size. Let us guess that the nucleic acid is first made: the egg before the hen. (The order is not important, protein could be made first.) This nucleic acid is then specifically attached to protein molecules already in the cell and, as the spacing of the nucleosides is rather flexible (Wilkins, private communication), this local attachment causes the opposite side of the nucleoside to form the same protein pattern, but in terms of bases or sugars.

The small proteins now being formed then find themselves able to attach to the bases or sugars *if* their over-all pattern fits the proper structure, which is that of the protein of the parent cell. Any other di- or tripeptides do not attach and probably suffer a new enzymatic digestion until they are reassembled in the correct way. Thus the nucleic acid serves to transfer the parental pattern to the new molecular generation.

Thus there rapidly develops a population of new protein and, in turn, this prints its design on new nucleic acid until the cell carries a high proportion of subunits ready for assembly.

The assembly process in the host follows the orderly development of the cell. In a virus-infected cell it conforms to the assembly needs of the virus.

All the above is speculative. It may be words and no more. It is the kind of basic thinking which will one day lead someone to the truth and we therefore include it with no great apology.

We need now to consider how the assembly of viruses from moderately large molecules can take place.

Forces Operative in Virus Multiplication

We have seen how the process of virus multiplication involves a dramatic change in the manufacturing processes of the bacterium so that the actual small pieces themselves are different, as exemplified by the virus antigens, which are small yet differ from the bacterial antigens. Although this is a most important fact, there is the equally important fact that in some way a virus, or part of a virus, can grow until it either divides or is the equivalent of two viruses. This requires an *ordered growth*, and the ordering process requires some kind of forces to be acting. We propose to consider here the kinds of forces which can be called on to explain ordered growth, and to make a speculative suggestion as to how they act.

There are essentially two ways in which an ordered array of matter can be made to develop. One is by the establishment of a concentration gradient of some kind, and subsequent diffusion. Thus if a cell is filled with small units of nucleic acid, and these are condensed into a solid phase at some point in the cell, there will be established a concentration gradient toward that point, and diffusion will occur. This diffusion will produce a steady accretion of nucleic acid at the condensation point. There is no doubt that this is an important feature of many cellular processes. However, the measured diffusion constants of protein molecules indicate that this process is far too slow to account for the very rapid virus multiplication. Accordingly, we turn to the second way, which requires the action of forces.

All forces between molecules are electrical in character, if we exclude very weak forces. They have their origin in the Coulomb field of a charged particle and owe their nature to the distribution of charge in the two systems which attract or repel each other. We have already seen that the first stage in virus attachment is electrostatic and must be due to the attraction of charges of opposite sign. If two elementary charges are involved, and they are separated by a distance r cm in a medium of dielectric

constant K, the mutual potential energy is $(4.8 \times 10^{-10})^2/Kr$ ergs. When r is 100 Å, and for $K = 80$, which applies to water for static or low-frequency fields, the potential energy is 2.9×10^{-15} erg. The energy kT, which is a representative thermal agitation energy, is 4.1×10^{-14} erg at 300° K, or 27° C. This is 14 times larger. Hence, such attractive forces are only capable of providing an energy able to hold a second molecule in place, in spite of thermal agitation, at distances of a few Ångstrom units or less.

It is, however, not necessary that a field be sufficient to account for a *binding* energy. If the field is sufficient to produce relatively rapid motion in a definite direction, this can cause two charges to come close enough together to produce binding. Now the electric field of a single, elementary charge in a medium of dielectric constant 80, at 100 Å, is 1,800 volts/cm. The measured electrophoretic mobilities of proteins are such that, in a field as high as this, a protein molecule can travel 100 μ/sec, or cover the 100 Å necessary to become bound in 10^{-5} sec. Thus forces due to free charges must be taken very seriously and probably account for some of the efficiency of assembly of molecular architecture.

The difficulty in the way of explaining the whole of the ordering of submolecular units in this way is the existence of ions in the bacterial cell. Whenever a free charge exists on the surface of a protein or nucleic-acid molecule, there will rapidly result an ionic atmosphere around this charge, and the consequent force at a distance becomes totally different. In fact, the most likely effect of the interaction of such charges and their atmospheres is a *repulsion*. We can now consider these ionic atmospheres, or *double-layer* forces.

Ionic Atmosphere, or Double-Layer Forces

For many reasons, a large molecule may either lose or gain surface charge and so acquire a net positive or negative charge. If this molecule now finds itself in a medium full of ions, the ions of opposite sign are attracted to the molecule and there results a *double-layer* as indicated in Fig. 8.6. The positive charges at the surface of the molecule attract negative ions,

which approach closely to the molecule. However, these, in turn, attract more positive charges behind them, and so an atmosphere results in which, *on the whole*, there is more negative charge near the molecule, but in a rather diffuse way, with positive charge mixed in with it. This is called a double-layer, which is an approximately correct description. The forces due to double-layers have been treated in colloid theory, notably by Verwey and

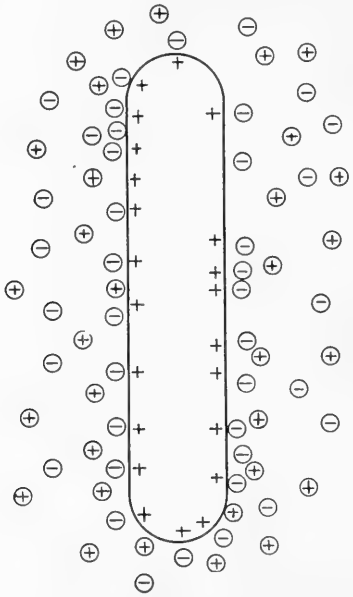


FIG. 8.6. The ionic atmosphere around a bacterium, drawn schematically to show the character of a double-layer.

Overbeek (1948). Two cases have been worked out in some detail—the cases of flat sheets and of spheres. A skeleton account of the theory is given here, less for the reason of deducing the results quoted than because it enables an understanding of the reason for the forces to be gained. A very clear general description of ionic processes and of the Debye-Hückel theory is given by Gurney (1946) which can serve as a background for the following material.

The double-layer is due to a fixed set of charges on the virus surface which influences a charge distribution in the solution con-

taining ions. This charge distribution is conditioned by the fact that an electrical potential energy is possessed by an ion near the virus surface, and this potential energy is a factor in the Boltzmann distribution of the ions. Thus, if we consider the virus surface to be a plane, there will be a potential V at a point near the plane, and so for an ion of charge multiplicity (valence) m, where the elementary charge is e, the ratio of the number of positive or negative ions, n_+ or n_-, per unit volume to the average number, n, is

$$\frac{n_+}{n} = e^{-meV/kT}$$

$$\frac{n_-}{n} = e^{meV/kT}$$

These are not the same, which is an elaborate mathematical way of saying that a double-layer will form.

Now the charge density, ρ, at any point can be calculated from n_+ and n_- and is simply

$$\rho = me(n_+ - n_-)$$

However, the charge density, ρ, is itself related to the potential by Poisson's equation:

$$\frac{\partial^2 V}{\partial x^2} + \frac{\partial^2 V}{\partial y^2} + \frac{\partial^2 V}{\partial z^2} = -\frac{4\pi\rho}{K}$$

so that we can, in principle, use these equations to calculate the electrical potential due to this ionic distribution. If we make some simplifications, which limit the range of operation of the result but do not fundamentally alter the theoretical processes involved, we can assume that all ions have the same valence, so m is the same (as indeed has already been written above), and can assume V is small, so that the first two terms of the exponential series are sufficient to describe the Boltzmann exponentials given above.

Then

$$n_+ = n\left(1 - \frac{meV}{kT}\right)$$

So

$$\rho = \frac{-2nm^2e^2V}{kT}$$

and therefore

$$\frac{\partial^2 V}{\partial x^2} + \frac{\partial^2 V}{\partial y^2} + \frac{\partial^2 V}{\partial z^2} = \frac{8\pi nm^2e^2V}{KkT}$$

Now for a plane surface which accumulates an ionic atmosphere, we need only consider the x-coordinate and, if we put $p^2 = 8\pi nm^2e^2/KkT$, we have the very simple equation

$$\frac{d^2V}{dx^2} = p^2V$$

for which the general solution is

$$V = C_1e^{px} + C_2e^{-px}$$

where C_1 and C_2 are constants determined by the physical conditions present. Since, $V = 0$ for $x = \infty$ we have $C_1 = 0$. And since $V = V_0$, the potential at the surface, for $x = 0$, we have

$$V = V_0e^{-px}$$

The double-layer potential has then dropped to 37% (or $1/e$) of its original value in a distance of $1/p$ cm, or a distance $\frac{1}{me}\sqrt{\frac{KkT}{8\pi n}}$. This depends sharply on m, the valence of the ions, and somewhat on n, the average concentration of the ions.

Now if two double-layers are formed at a distance $2d$ apart, there will be an interaction between them which will result in a repulsive force and hence a net potential energy which is a function of $2d$, their separation. It is possible to calculate this repulsive potential, V_R, in several ways (see Verwey and Overbeek, 1948, p. 66), and an approximate value for this is

$$V_R = \frac{64nkT}{p}\left[\frac{e^{p/2} - 1}{e^{p/2} + 1}\right]^2 e^{-p2d}$$

An approximate value for the force, F, between two such plate double-layers is

$$F = 64nkT\left[\frac{e^{p/2} - 1}{e^{p/2} + 1}\right]^2 e^{-pd}$$

This force depends on the concentration of ions and also on p. The greater the value of p, the more rapidly does the force fall off with distance.

This type of force is remarkably interesting from a biological viewpoint, and it is undoubtedly one of the factors in the organization of large molecules into the pattern of a virus or a chromosome. A living cell which is metabolizing is the seat of rapid energy turnover, of rapid molecular change, and of synthesis. It is surrounded by a membrane capable of selective passage of ions. As a result, the ionic strength of the inside of a cell is continually changing as the development of the cell proceeds. The expression for the force between two large molecules (which, for the present, we can think of as represented by planes) is dependent critically on the ionic concentration and also on p, which contains both the concentration and the valence. Thus the degree of repulsion between two large molecules is *under control by the condition of the cell.* We shall see shortly that attractive forces of a different character exist, and the balance between these will determine whether two large molecules remain near each other or will be repelled apart. We have seen that Luria's mutation studies indicate that virus multiplication consists of an exponential growth involving something like a growth, division, and separation. The separation is almost certainly constrained to take place because of the repulsive force due to ionic atmospheres.

Evidence for this repulsion as a factor in virus action was obtained by Bernal and Fankuchen (1941) in their X-ray studies of tobacco mosaic virus.

The preparations of this virus showed strong birefringence, and the orientation causing this persisted even on drying. In order to study the process, they constructed a small-angle X-ray diffraction camera, using slits in place of circular openings in

order to observe the reflections corresponding to the large separations between whole virus particles. They found sharp reflections and were able to make accurate measurements of the interparticle distances. The X-ray diffraction pattern corresponded to a hexagonal, close packed array.

The interparticle distance varied with the virus concentration in the manner shown in Fig. 8.7. The interparticle distance R, in Å, was found to be $R = 1{,}650/N^{1/2}$, where N is the number of

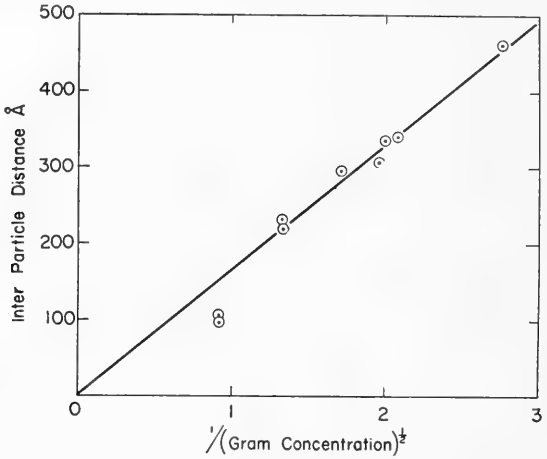


FIG. 8.7. Variation of the intervirus distance for TMV at various concentrations, as observed by Bernal and Fankuchen (1941). The fact that a linear relation with the reciprocal of the square root of concentration is observed means that a close packed array exists.

grams of dry virus per 100 cm^3. The plot in the figure is R versus the reciprocal of the square root of the concentration. This separation into a hexagonal array can be explained by a repulsion between the particles, causing them to fit the space in the container with as much distance between particles as possible. The results have been analyzed by Oster and Onsager and can be fully explained by repulsive forces only.

The repulsive potential due to the double-layer interaction for two spheres, radius a, can be calculated for a variety of cases. For a thin double-layer, corresponding to a moderate ionic concentration (about one-tenth molar for example),

Verwey and Overbeek give the approximate value for the repulsive potential, U_R, as

$$U_R = Ka\psi_0^2[\tfrac{1}{2} \ln (1 + e^{-\tau(s-2)})]$$

where K is the dielectric constant; ψ_0 is the surface potential; which is of the order of 30–150 mv, corresponding to 4.8×10^{-14} erg; τ is pa or $[8\pi nm^2e^2/KkT]^{\frac{1}{2}}a$; and s is the ratio of the separation of the centers of the spheres to the radius of each.

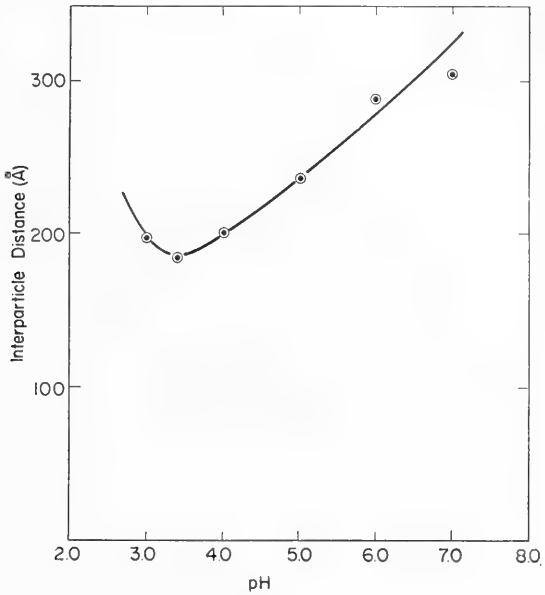


FIG. 8.8. Variation of intervirus distance with *p*H for TMV. A minimum occurs at the isoelectric point, which indicates that forces exist which are dependent on ionic atmospheres. Data due to Bernal and Fankuchen (1941).

The fact that the force between virus particles is dependent on the ionic atmosphere is shown by the way in which interparticle distance for TMV was found by Bernal and Fankuchen to depend on *p*H. This is shown in Fig. 8.8. It can be seen that there is a minimum distance at the isoelectric point, *p*H 3.4, with a rise on each side.

It is quite clear that repulsion alone cannot explain the assembly of macromolecules to form a virus unit. Some kind of

attraction is necessary. Two types of attractive processes exist, and probably both play a part in virus synthesis.

Van der Waals Forces Between Macromolecules

The forces between neutral molecules which give rise to liquefaction of even spherical molecules like helium, and which are known generally as Van der Waals forces, have been explained by London (1930, 1937). According to quantum mechanics, an electron associated with a positive nucleus to form an atom does not occupy a smoothly regular orbit, but has a chance of occupying many different positions with respect to the nucleus. Thus, although the average location of the electron may be such that no net-time-averaged dipole moment exists, there may be very large instantaneous dipole moments, and a mean-square dipole moment may have a considerable value.

Now each ephemeral dipole produces an electric field around it, distorts nearby atoms, and produces induced dipoles in them. These induced dipoles now interact with the originating transient dipole and cause an attraction. There are thus two factors to consider—first, the size of the transient electric field due to the random position of the electron in the first atom, and second, the readiness of the second atom to change its configuration to produce a dipole and so become attracted. Once the originating and induced dipoles have assigned values, the mutual potential energy due to their interaction (which is attractive) is easy to compute. The electric field due to a dipole varies as $1/R^3$, so the induced dipole will have a strength dependent on this, and the attractive potential between two dipoles also depends on $1/R^3$, so that the over-all attractive potential depends on $1/R^6$.

The magnitude of the constant of proportionality depends on the polarizability,* α_2, of the second atom and the polarizability, α_1, of the first. In addition, if the frequency for an electronic transition from a state L to a state K, of energies F_L and E_K is ν_{kl}, so that

$$h\nu_{KL} = E_L - E_K$$

* Polarizability is the induced dipole moment per unit applied electric field.

and ν_0 is the lowest possible frequency of such a transition, London deduces that the factor $(3/4)h\nu_0$ is also involved. The mutual potential energy, U, is then

$$U = -\frac{3h\nu_0\alpha_1\alpha_2}{4R^6}$$

Actually this calculation is difficult to make, and other versions exist. In general we can put $U = B/R^6$, where B is a constant for any pair of atoms.

From our point of view, two vital considerations exist. The first is that these forces are *additive*, so that one fluctuating atomic dipole influences and attracts all other polarizable atoms. The second is that the size of viruses on the molecular scale is large, and the electric field accordingly takes time to travel to all points in the virus, a time which is admittedly very short but not short compared to the rate of fluctuation of the inducing dipole. This means that remote atoms in the virus may be influenced by a field which does not correspond to the existing dipole and so will be out of phase with it and thus much less attracted. The first consideration leads to the remarkable result that between large molecules the attractive potential diminishes quite slowly with their distance apart, d; in fact, over a small range, the potential varies as $1/d^2$. The second consideration limits the range in which forces can be treated as additive and, indeed, may require that repulsive forces be considered. So for distances exceeding 100 Å, the attractive potential varies more rapidly with distance and, in fact, falls very quickly in value.

The forces between plane surfaces of infinite extent and finite thickness, neglecting the finite-velocity effect mentioned above, were calculated by de Boer (1936), and Hamaker (1937) has considerably extended the calculation to include spherical particles. For the case of two parallel plates of thickness y, the additive feature of the forces is readily exploited, and by two quite simple integrations (see Verwey and Overbeek, 1948, p. 101) an expression for the potential, U, can be derived. It is

$$U = \frac{\pi N^2 B}{48}\left[\frac{1}{d^2} + \frac{1}{(d+y)^2} - \frac{2}{(d+y/2)^2}\right]$$

where N is the number of atoms per unit volume of the plane material, and B is the constant in the relation for the potential energy of two atoms.

In the case of two spheres, radius a, placed so that the shortest distance between their surfaces is H, we can use the parameter $S = \frac{2a + H}{a}$. Thus S is the ratio of the total separation of the centers to the diameter of one sphere. Then the resulting potential energy (Verwey and Overbeek, 1948, p. 160) is

$$U = -\frac{\pi^2 N^2 B}{6}\left[\frac{2}{S^2 - 4} + \frac{2}{S^2} + \ln\frac{S^2 - 4}{S^2}\right]$$

or, approximately,

$$U = -\frac{\pi^2 N^2 B a}{12H}$$

For a rough guide, we can follow Verwey and Overbeek and put $\pi^2 N^2 B = 10^{-12}$ ergs, so we have

$$U \sim 9 \times 10^{-13} a/H$$

In actual cases, the macromolecules are immersed in a medium which intervenes between them. This does not, however, diminish the field strength except in the usual way in terms of the dielectric constant. At the frequencies involved, there is no opportunity for wholesale rotation of polar molecules but only for the electronic adjustments in each. Thus the dielectric constant to be used is the square of the refractive index [about $(4/3)^2$]. So the effect of the medium will be to modify downward, by a factor of about two, the value of the interaction potential.

Fluctuating Proton Charge Forces

One of the characteristics of proteins is the fact that many of the side chains consist of amino acids containing dissociating groups. Groups such as $—NH_2$ can attach a hydrogen ion to become $—NH_3$, and a group like $—COO^-$ which has already lost a

hydrogen can reattach one to increase the local positive charge. In the ordinary way, there are many of these groups and not a great deal of reason why one should have hydrogen attached to it, rather than another. The charged groups produce dipole moments which, on the whole, average to a small value, but which can, by fluctuations in the positions of the attached protons, cause quite large mean-square dipole moments. Kirkwood and Shumaker (1952) have pointed out that such fluctuating dipoles can influence the migration of protons in a second protein molecule and thus induce dipoles which are attracted to the first molecule. If one were constructing a large-scale model to illustrate the nature of the London-Van der Waals forces, this would serve very well; the reason for the fluctuations is totally different, and the factors influencing their size are also quite different, but the net effect of producing attraction is the same.

The theory developed by Kirkwood and Shumaker can be used to explain the fact that small concentrations of protein in solution produce a large change in dielectric constant, a fact which was previously ascribed to the presence of large, permanent (or nearly so) dipole moments. If, instead of such moments, there is supposed to be a lability of the protons which are attached to the kind of groups just described, then the influence of an external electric field will be to modify the proton distribution and bring forth induced dipoles, which are large, on the average, and so cause an increase in the dielectric constant. For the four proteins β-lactoglobulin, ovalbumin, hemoglobin, and serum albumin, the mean-square dipole moment found experimentally agrees quite well with the theoretical predictions.

In view of this fact, the value for the interaction energy between protein molecules calculated by the same authors is of considerable interest. If U is the interaction energy

$$U = \frac{(\overline{\Delta q_1})^2 (\overline{\Delta q_2})^2}{2K^2R^2kT}$$

where $(\overline{\Delta q_1})^2$ and $(\overline{\Delta q_2})^2$ are the average, total charge fluctuations of the two molecules. The energy falls off as $1/R^2$, which is very

similar to the rate of change of energy for the Van der Waals forces between large molecules.

A force of this nature is dependent on the average charge fluctuations in the molecules, and these will, in turn, depend on conditions in the solution which attach or detach protons from ionic groups. Therefore, the condition of the molecule can have a considerable effect on this variety of force.

This kind of force is not inherently specific; that is, it does not depend on the presence of similar groups or complementary structures. However, the interaction energy may well be increased in value if certain kinds of complementary groupings exist in the two molecules. Kirkwood and Shumaker point out this fact, but make no definite calculation of interprotein forces numerically.

We now have three classes of force to consider, one repulsive and under metabolic control by changing the ionic strength and one attractive and also dependent on whatever cellular conditions determine the proportion of charge subject to fluctuation. The third force, the Van der Waals force, is not under control by the nature of the solvent in the cell at all.

Potential Energy Diagrams for Two Macromolecules

There is not a great deal of value in trying to plot accurate potential diagrams for the interaction between two biological molecules since neither the size nor the structure of these molecules is accurately known. Nevertheless, it is worth while to get some idea of the order of magnitude of the potential energies involved so that suggestions for the physical processes in virus multiplication can be made.

To make a definite case, in Fig. 8.9 we plot the potential-energy curve for two southern bean mosaic virus particles according to the attractive-force formula of Hamaker and the repulsive-force formula given by Verwey and Overbeek. The value of the surface potential, ψ_0, has been chosen quite arbitrarily as kT/e for a temperature of 300° K. It can be seen that the potential energy, U, is slightly negative at large distances,

which means there is a small attraction between the particles. This is not significant because thermal agitation is sufficient to prevent any important binding between virus particles at this range of separation. As the distance apart becomes less, there

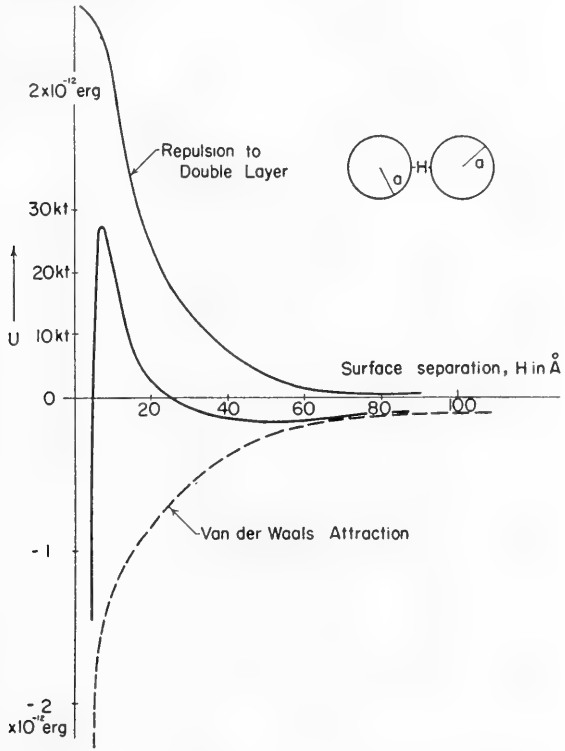


FIG. 8.9. Potential energy between two southern bean mosaic virus particles. The double-layer repulsion and the Van der Waals attraction produce a potential barrier. This varies in character with ionic strength, and may be a factor in keeping constituents together during growth and then forcing them apart at division.

is a sharp increase in the mutual potential energy, which means a quite definite repulsive force in this region. At a distance of about 6 Å, the Van der Waals attraction makes up for the double-layer repulsion, so that within a range of 6 Å there is quite strong binding between the two particles.

We can now imagine a process about as follows. The original virus invades the host cell, and probably a long nucleoprotein molecular aggregate unwinds and presents a large area of specifically active surface. This starts a strongly competitive system of enzyme manufacture, and possibly also of direct enzymatic action, which sets going a new process of synthesis of virus precursor material, both nucleic acid and protein. The nucleoprotein units so formed become attracted by Van der Waals attrac-

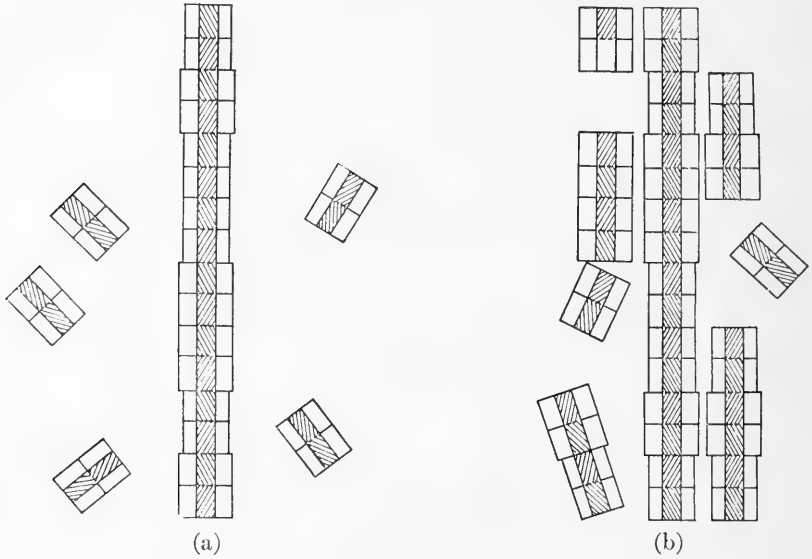
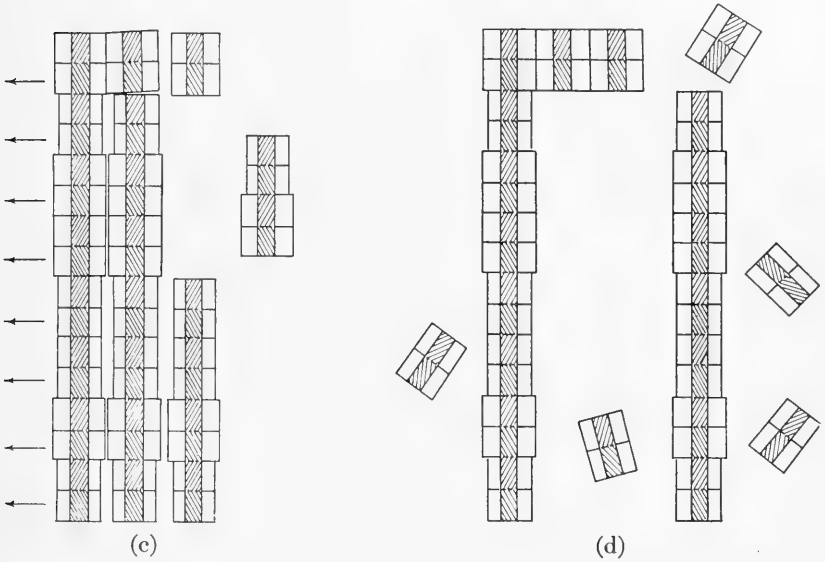


FIG. 8.10. Token representation of the process of self-duplication. The nucleoprotein units are attracted to an original chain, aggregate near it to form adjacent chains, experience a change in ionic atmosphere, and are forced

tion and held in place so that near the original unit a second set of nucleoprotein molecules accumulates. This will grow, but only within the 6-Å attractive distance, so that a second, long, thin nucleoprotein is formed. This will continue to thicken and develop as the metabolic processes of the host pile up more nucleoprotein until the thickness of both original and new nucleoprotein exceed about 200 Å. Under these conditions, the Van der Waals attraction may begin to lessen on account of the fact that the additive feature of the intermolecular forces may cease

to hold because of the finite velocity of travel of the electric field. Thus if the effective frequency of an electronic fluctuation is 10^{16} per sec, the time for the field to be established at a distance of 100 Å is the distance divided by the velocity of light, or $10^{-6}/3 \times 10^{10}$, or 3.3×10^{-17} sec. This means that the induced dipole will not be in phase with the original fluctuation dipole and no attraction will occur. So this may well mean that the



apart to form two chains. Part of one chain can go with the other at this stage, and the second chain can be completed from a unit near by.

attraction to the original nucleoprotein chain is too little to hold within the 6-Å critical distance. As soon as this is exceeded, the strong repulsive forces of the ionic atmosphere take over, and the two units are forced apart, ready to start a second pair of chains, and so on.

It is interesting, though speculative, that the size of a genetic unit is thus restricted by the finite velocity of light, so that ultimate cosmological laws can be seen influencing the intimate details of a living system.

The kind of process envisaged is shown in Fig. 8.10. The center, internally active part of the virus is thought of as long and thin and comprising nucleoprotein units joined together by a mixture of Van der Waals forces and hydrogen bonds. The units are firmly, but not inseparably, bound. This long, thin unit offers a large surface area to the metabolizing bacterium and, as a result, rapidly changes the molecular environment so that fresh nucleoprotein begins to be formed. As this takes place, the immediate neighborhood begins to be populated by subunit nucleoproteins, each capable of influencing protein and nucleic-acid synthesis as described earlier. These are attracted to the original nucleoprotein and also to one another and begin to form alongside the first virus element as shown in Fig. 8.10b. As they aggregate to form new lines along the old virus, the ionic atmosphere changes to make possible a repulsion. As soon as there are several lines formed on each side of the parental virus, the Van der Waals attraction ceases to be enough to overcome the ionic repulsion, and the new units are driven out. Some are complete and some are not. Each unit now starts its double process, of charging the metabolic synthesis mechanism and of mutual accretion.

This process will develop exponentially, and before long the cellular metabolism will have become almost wholly a virus metabolism contained in the cellular envelope. At a stage, which is about three-quarters of the whole duration of single cell development, the nucleic acid synthesis becomes essentially complete. A further protein synthesis continues until the virus particles are covered with a protein sheath which includes the enzymes necessary to produce cellular lysis and entry in the first place. These are able to produce lysis in any event and, in the case of bacterial viruses, do so, liberating a burst in the well-known way.

We have mentioned that some incomplete virus chains will be formed. To some extent, these must remain until the burst occurs and must be part of the whole debris which is found at that time. However, there must also be some over-all checking process, as suggested by Dancoff (1949), which renders the multiplication of smaller units unfavorable. Perhaps this can be found

in more specific attractive forces due to special arrangements of charges on the surfaces of the virus chains which act to make the attraction of subunits considerably less unless the whole chain is complete.

It can be seen that the multiple mating process falls into this scheme. Each genetic unit is thought of as being one of the nucleoprotein building blocks shown in Fig. 8.10. It is quite easy for the separation of two chains to carry away part of the first; in fact, the whole process is one of ordered assembly of separate units rather than of the splitting of one individual into two halves. So it is not surprising if a genetic unit which started in one chain finds itself part of another chain.

It is also clear that the generations need not be synchronized, but only approximately so.

This last part of this chapter has been frankly speculative. In truth, all that can be said is that we have some small idea of the probable nature of the virus precursors, that there exist attractive forces between large molecules up to a certain size, and also repulsive forces due to ionic atmospheres, which are somewhat controllable by the cellular state. What has been done above is to try to invent a scheme using these ideas.

The interest and value of such a scheme is not essentially in its truth but rather in its suggestiveness. We have said before that virus multiplication may form an elementary test of the adequacy of physical laws to explain fully biological processes. Using some simple facts regarding large molecules in solution, it can be seen that known physical laws contain at least some of the necessary features demanded of them. This is encouraging, but it remains for the virologist to provide fuller information about the parts and pieces and the biological processes, for the virus physicist to make their information fully quantitative and handy for accurate thought, and for the theorist to put all the information correctly together to see whether known natural laws can accurately interpret and predict events in virus development.

Physical studies of viruses aid in this goal, and it is because they aim toward a solution of an outstanding problem of humanity that even preliminary steps, as we have described here,

are worth description. Far greater development of these studies is needed and is going forward. It is with a keen eye to the promise of the future that we conclude this chapter.

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