# VIRUSES

*BIOLOGY*
*COLLOQUIUM*
*1950*

OREGON STATE CHAPTER OF PHI KAPPA PHI
OREGON STATE COLLEGE • CORVALLIS • 1950

*Eleventh Annual Biology Colloquium*
*Saturday, April 29, 1950*

# Viruses

OREGON STATE CHAPTER OF PHI KAPPA PHI
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WENDELL M. STANLEY, Ph.D.

*Leader of Eleventh Annual Biology Colloquium*

# FOREWORD

The Biology Colloquium is conducted in a spirit of informal discussion and provides opportunity for participation from the floor. The colloquium is sponsored by the Oregon State Chapter of Phi Kappa Phi with the collaboration of Sigma Xi, Phi Sigma, and Omicron Nu. Sigma Xi assumes special responsibility for the colloquium luncheon. Phi Sigma and Omicron Nu provide afternoon tea. The College Library arranges special displays of the writings of colloquium leaders and notable works on the colloquium theme.

Grateful acknowledgment is made of the cooperation and interest of the several faculties of Oregon State College that are concerned with biology, of those biologists contributing to the program, of Chancellor Paul C. Packer, President A. L. Strand, and other executives of Oregon State College.

The first Biology Colloquium was held March 4, 1939, with Dr. Charles Atwood Kofoid of the University of California as leader, on the theme "Recent Advances in Biological Science." Leaders and themes of succeeding colloquia have been: 1940, Dr. Homer LeRoy Shantz, chief of the Division of Wildlife Management of the United States Forest Service, theme "Ecology"; 1941, Dr. Cornelius Bernardus van Niel, Professor of Microbiology, Hopkins Marine Station, Stanford University, in collaboration with Dr. Henrik Dam, Biochemical Institute, University of Copenhagen, theme "Growth and Metabolism"; 1942, Dr. William Brodbeck Herms, Professor of Parasitology and Head of the Division of Entomology and Parasitology, University of California, theme "The Biologist in a World at War"; 1943, Dr. August Leroy Strand, Biologist and President of Oregon State College, theme "Contributions of Biological Sciences to Victory"; 1944, Dr. George Wells Beadle, Geneticist and Professor of Biology, Stanford University, theme "Genetics and the Integration of Biological Sciences"; 1946, Dr. Robert C. Miller, Director of the California Academy of Sciences, theme, "Aquatic Biology"; 1947, Dr. Ernst Antevs, Research Associate, Carnegie Institution of Washington, theme, "Biogeography"; 1948, Dr. Robert R. Williams, Williams-Waterman Foundation, theme "Nutrition"; 1949, Dr. Eugene M. K. Geiling, Head of the Department of Pharmacology, University of Chicago, theme, "Radioisotopes in Biology." Because of wartime travel conditions, the 1945 Biology Colloquium was omitted.

# Eleventh Annual Biology Colloquium

*Theme:* VIRUSES

*Leader:* WENDELL M. STANLEY, Ph.D., In Charge of the Virus Laboratory, University of California

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# Biochemical Studies on Plant Viruses

WENDELL M. STANLEY

MR. CHAIRMAN, President Strand, Ladies and Gentlemen: I am not so sure of my ability to bring viruses out of chaos. Many times, in discussions, it seems to me as though we are almost back in Aristotle's time, about 3,000 years ago.

This morning I have elected to tell you something of the story of plant viruses. This evening after dinner when we shall have had a full day of facts and figures presented to us, I hope to take up the thought expressed by President Strand in connection with the general problem of biogenesis, and perhaps philosophize a bit and talk about things that do not lie at the moment within the realm of fact or proof. I hope that will provide a suitable after-dinner discussion.

It is a comparatively easy thing to go back to the beginning of viruses because the first virus was discovered in 1892, just a little over fifty years ago. This discovery was made by a Russian and was really a first—a Russian botanist by the name of Iwanowski, working with an ordinary disease, the tobacco mosaic disease. The experiment which he conducted was a very simple one of grinding up the leaves of the diseased plant, pressing out the juice, and passing it through a so-called bacteria-proof filter. For those of you who are not working intimately in the biological field, I might state that it is somewhat like a kitchen colander with the usual holes except the holes are so very tiny that they retain ordinary bacteria. Iwanowski found that the material which was passed through these very tiny holes which retained bacteria would, when applied to a normal tobacco plant, cause that plant to come down with the disease. Unfortunately, Iwanowski refused to believe his experimental evidence because he was quite convinced that this so-called bacteria-proof filter did in fact have holes and he, by virtue of refusing to accept experimental evidence, in my estimation at least, failed to make a discovery. He did not recognize this as a new type of infectious agent. He continued to believe that this infectious agent was simply due to bacteria which had passed through the holes in the filter, despite the fact that he had tested the filter many times against known bacteria and the filters had retained such bacteria. In other words, he refused to believe his experimental data. That was due, of course, to the fact that during the preceding forty years or so, bacteria had been thought to be responsible for this, that, and the other type of disease. In other words, the so-called "Golden Era of Bacteriology" was still in full force. He was apparently so imbued with the thoughts of the times that he chose to disregard his experimental evidence. As far as I am concerned, the real discovery of viruses was made by a Dutch botanist by the name of Beijerinck just six years later, in 1898. Beijerinck was, undoubtedly, much the more clever of the two men because he immediately recognized the possibility of his having made a major discovery. He recognized the possibility of the symptoms being caused by a toxin and not by an infectious agent. To eliminate that possibility, he carried on such a filtration experiment in a series. He would grind up the plant, press out the juice, pass it through a filter, apply the filtrate to a healthy plant, and this plant would come down with the disease. He would grind up the leaves of that plant, and pass it through another filter, and so on for as many transfers as you wish. That eliminated, of course, the possibility that the infectious agent—or the symptoms—resulted from a toxin.

Beijerinck had had some chemical training, and performed a rather large number of quite significant experiments. He ended up, though, by deciding quite definitely that he had discovered a new type of infectious agent. He called this agent a "contagious living fluid." If you analyze the words, I think you will see right away that he was not thinking in terms of bacteria or of anything like bacteria. In speaking of a living fluid, one may—at first thought—think that one word is a little in opposition to the other. As far as I can see, however, we do not have a better description even today. After Beijerinck's discovery, the general characteristics of viruses began to become fairly well differentiated. Obviously, from the fact that they would pass through these so-called bacteria-proof filters, they must be very small; in other words, viruses in general are smaller than the bacteria or other types of ordinary living agents—and that has continued to represent one of the major characteristics of viruses. The fact that they can reproduce or grow, of course, is typified by their infectious nature. They are very small, and they can reproduce, and they can grow.

A third important characteristic is that during their growth, or reproduction, they have the ability to do what we refer to as mutate. In other words, they can change or adapt themselves apparently to new surroundings or new conditions so that they give rise to a slightly different type of disease. Another characteristic of viruses is their great specificity. A given virus will usually grow or reproduce only within the living cells of certain specific hosts. We have viruses, for example, which are so highly specialized that they will grow only in a particular kind of cell in a particular animal. The Shope rabbit papilloma virus, for example, will grow only in certain rabbits and there only in the skin tissue of the rabbit. If you tattoo it into the skin across the mucous membrane of the lip, it will take only in the skin and not even in that tissue represented by the mucous membrane.

There are two minor characteristics related to the over-all virus picture. One is that a great many, but not all viruses, produce a lasting immunity. You will recognize immediately common childhood diseases, such as measles and mumps and chickenpox, which one usually has but once; having had them, one usually has a lifetime immunity. But you might think immediately of certain other virus diseases, such as the common cold and influenza, where the immunity is apparently a transient affair and lasts in some cases only a few weeks or a few months or at most a very few years. Another characteristic of certain viruses is the fact that their activities result in the formation of inclusion bodies, sometimes in the nucleus and sometimes in the cytoplasm. We have examples, in the plant viruses, of both types.

These, then represent the characteristics of viruses which were noted, in some cases, quite early and which have been found to hold down to the present day. Now I should like to give you an idea of the sizes of viruses. A chart can be drawn which covers the range from the large viruses, such as vaccinia, all the way down through to the very small viruses. At this level some viruses overlap with certain protein molecules. Prior to 1935, about all that was known of viruses was the fact that the viruses, in general, were smaller than bacteria; that they were infectious, and that they did tend to mutate. Concerning their basic nature, however, practically nothing was known. It was not known whether they were smaller ordinary living organisms, or whether they represented some new type of entity. There was considerable discussion, during those years, concerning the probable nature of viruses. Many people thought they were protein in nature; other people thought they were carbohydrate in nature; still other people thought they were fatlike in nature; and, of course, the main discussion centered around the question whether they were living organisms or nonliving entities.

In order to resolve this situation, which was really chaotic in those days, it became necessary to collect and purify one of the viruses and make an attempt to learn something about it. The one selected was one which is in the center of the group with respect to size, the tobacco mosaic virus. In order to work with a virus, it is necessary to devise some means of measuring the amount of the virus present. This is a point that should be emphasized very strongly because it applied then and it applies today, as one searches for new viruses. If one wants to work with the viruses, it is almost absolutely necessary to be able to measure virus activity. Fortunately at about the time this work was started, a so-called local lesion method of measuring tobacco mosaic virus was discovered. This technique involves simply rubbing a solution or an extract of a virus material over the upper surfaces of the leaves of certain plants which will respond with local lesions. It was found that the number of lesions produced could be used as a measure of the amount of virus which was present. These spots come out in about forty-four to forty-eight hours and, if you set up your experiment correctly, it is possible to measure tobacco mosaic virus with an accuracy of about 10 or 15 per cent. That is a remarkable accuracy for a biological test. It approaches, for example, the accuracy, usually given as approximately 3 per cent, for the ordinary Kjeldahl determination for nitrogen; but this is a biological test in which you simply rub the virus over the upper surface of the leaves and then measure or count the number of spots. Doing that over a range where there is a straight line relationship and using leaf pairs, proper age of plants, etc., you can measure tobacco mosaic with an accuracy of 10 to 15 per cent. It was solely this local lesion method which made it possible to conduct the vast amount of biochemical or physico-chemical work which has been carried on with this virus during the past fifteen years.

Using this measuring technique, it became possible to conduct a variety of experiments. Much time was spent on determining the effects of a variety of chemicals on extracts of mosaic virus from diseased plants. It was soon found

that protein-precipitating agents always precipitated the virus activity. It was found that oxidizing agents would inactivate or destroy the virus activity. It was found that strong acids and strong alkalis inactivated the virus, and did so irreversibly. It was found that different viruses had different pH ranges of stability; in other words, if you made extracts of a variety of viruses and subjected them to different hydrogen ion concentrations, each virus had a particular range over which it would retain activity. If you made it more acid or more alkaline, the activity would disappear. Tobacco mosaic is stable between about pH 2 and pH 8. If you go more acid or more alkaline, it loses activity. Certain other plant viruses have somewhat narrower pH ranges of stability. It began to look as though you had different types of entities which were susceptible in varying degrees to different acidities or alkalinities. The work with chemicals indicated that the protein-precipitating agents tended to precipitate virus activity. We therefore subjected these extracts to the action of certain proteolytic enzymes.

In work with pepsin and tobacco mosaic virus at pH 4, 5, 6, 7, and 8 there was very little change in the virus activity with time. It so happens that pepsin is inactive as a proteolytic agent at those same hydrogen ion concentrations; but at pH 3, where pepsin begins to be fairly active as a proteolytic agent, there was a sharp decrease in the virus activity—indicating that the proteolytic enzyme, pepsin, was hydrolyzing a protein which was either a virus or closely associated with, and necessary for, virus activity.

If you decrease the amount of pepsin you decrease the rate, or if you increase it you increase the rate. Of course, if you subject it to a below-freezing temperature so that you do not have a solution, this should interfere with the rate of hydrolysis and this is what happens. If the inactivation were due to some absorption or inhibition or something of that type, then you ought to get this decrease in activity regardless of the temperature. Other experiments demonstrated that there was nothing in the digestion mixture which was inhibiting virus activity.

Recent experiments have been fairly important because they have provided the first definite information that this particular virus either is—or is closely associated with—a protein. Of course, this does not mean too much in a way because we know that living organisms have protoplasm consisting of proteins but, in general, they are resistant—you have to kill them before you can get action with enzymes. The experiments were important back in the early days simply as a means of bolstering an idea—that this virus might conceivably be some sort of a protein. Following these experiments, we then went into the use of the ordinary methods of protein chemistry consisting of an attempt to get isoelectric precipitation through the use of high concentrations of salt, like ammonium sulphate or sodium chloride. In a comparatively short time, as a matter of fact almost surprisingly so, it was possible to isolate from the juice of mosaic-diseased plants a material that crystallized in the form of long needle-like crystals. This material has turned out to be a nucleo-protein, having an unusually high molecular weight. The individual particles which go to make up the long needle-like crystals have turned out to have some rather interesting properties. The immediate question which came up, of course, is: What is the nature of the material which could be obtained in the form of the crystals? It was necessary to study solutions of this material and this was done by means of the ultracentrifuge.

Very early, we ran into some interesting things such as two sedimenting boundaries. That was an observation that was made back in 1937. We did not have an explanation back in those days; now, we do have. We know that the second boundary represents an aggregation, end to end, of two particles of this particular virus. There has been quite an interesting story concerning that type of aggregation. This early experiment, though, showed that whatever we had present in this purified material, this purified nucleo-protein, was relatively homogeneous from the standpoint of centrifugation. Another way of testing homogeneity is, of course, to subject the nucleo-protein to an electrical field under different hydrogen ion concentrations and, under the influence of the electrical field, you can move the particles of nucleo-protein. You simply have a solution of the virus and form the boundary between it and the solvent and apply an electrical field. The particles act just as though you started out a platoon of soldiers who were perfectly trained and you marched them forward a hundred steps and then backwards two hundred steps and then forward a hundred steps. They return exactly where they started from and the particles do likewise only if they have a remarkable degree of electrical homogeneity. Such experiments demonstrated that this material was remarkably homogeneous from the standpoint of its electrical charge. Of course, all during this time there was that big question in our minds: Is this nucleo-

protein tobacco mosaic virus? I can assure you that a great many people were very vehement about this particular question. Some were quite convinced that it was not tobacco mosaic virus and a great many people thought that it had nothing to do with tobacco mosaic virus. It was necessary, therefore, to get experimental evidence which might be used to answer the argument as to whether or not the nucleo-protein was or was not the virus. About the only way to do that was by a variety of so-called correlation experiments in which you do something to the protein and then measure it and see what has happened to the virus activity. The basic concept of this is all very simple—it is just as simple as when I talk here at first and then move over to this spot. You see me here and you hear me talking from here, and then you decide that I have moved over. Everything associated with me has moved over; there is nothing left at the former position. The basic idea we are considering—and there is nothing very complex about it even though some of the experiments did get rather complex—is just as simple as that. You do something to the nucleo-protein, either move it physically or do something to it chemically, and then you determine the nature of the biological activity. In a particular experiment, all we did was to have a series of solutions of this purified nucleo-protein at different hydrogen ion concentrations, and we measured the activity. At the same time we determined its sedimentation characteristics. It was found that at somewhere between pH 2 and 8 the sedimentation constant remained in the neighborhood of 194, meaning that the integrity of the particle was unchanged. In other words, as you sediment the material or subject it to certain hydrogen ion concentrations, the activity is still there. But consider what happens at hydrogen ion concentrations more acid than pH 2. We found that the activity dropped off precipitately at the same pH, approximately, that the high molecular weight nucleo-protein literally fell to pieces to give low molecular weight material. The same thing happened at about pH 8, although the breakdown was not so rapid on the alkaline side. Therefore in acids and in alkalis this large molecular weight material disintegrated as shown by sedimentation work at exactly the pH at which the activity disappeared. Well, you may say, What of it? This merely means that this is one type of correlation experiment. That is what should happen, of course, *if* that nucleo-protein actually represented tobacco mosaic virus. However another type of experiment was made to show what happens when you allow a solution of the material to stand in six molar urea which is a protein denaturing medium. In this case one of the activities followed was double refraction of flow which this particular virus exhibits. Another property followed was the turbidity and another the formation of insoluble protein. The activity drops off more rapidly than the other properties but in general the various properties follow one another fairly closely. It means that, under these conditions, the biological activity and the integrity of that nucleo-protein are essentially identical. I do not expect to go on and describe correlation experiment after correlation experiment, despite the fact that we spent about ten years doing just this sort of thing. Many types of such experiments have been done with tobacco mosaic, with tomato bushy stunt virus, with influenza virus, vaccinia virus, and a variety of other viruses. These were subjected to inactivation with various types of energy, with ultraviolet light, with X-rays, with partial hydrolysis by enzymes, and with a variety of other chemical agents. In every case, when you destroy the integrity of high molecular weight nucleo-protein you destroy virus activity. We concluded some years ago that the nuclear protein was in fact tobacco mosaic virus and that the virus activity was an integral part of and came directly from the nucleo-protein. Of course, when you once arrive at that conclusion you tend to taper off on correlation experiments and go on to things of more importance.

Before discussing some of these things that I consider more important, I should like to discuss a very interesting phenomenon that is characteristic of tobacco mosaic virus. In England Bawden and Pirie in conjunction with Bernal made the observation that, if a tube of the purified virus is allowed to stand, it separates out into two layers. If you look at it with crossed Nicol prisms, you can see that the lower layer is spontaneously birefringent. That was very interesting; it had not been known before except in the case of highly symmetric things, such as vanadium pentoxide, bentonite, etc. This apparently means that in the lower layer you have a type of crystallization. Sometime prior to this, two plant pathologists on the Berkeley campus, Takahashi and Rawlins, noted that solutions of tobacco mosaic virus, even unpurified solutions, would exhibit what is known as double refraction of flow. They noted that if you simply allow the solution to flow from a pipette and examine it with crossed Nicol prisms or polaroid plates, you

find the entire flowing solution is doubly refracting. They reasoned that because the entire solution is doubly refracting the solution must contain rod-shaped particles. That, then, represented the first indication that this nucleo-protein had an unusual shape. Experience subsequent to that time has fully confirmed these early observations. This apparently stems solely from what we call form double refraction. In other words, the nucleo-protein itself, as a particle, apparently has no intrinsic crystallinity because, if you prepare solutions of this material in solvents having different indices of refraction and then make these flow and attempt to measure stream double refraction, you find that it starts out with a high double refraction of flow because you have solvents which are quite far in their index of refraction from the index of refraction of the nuclear protein itself. As you approach an index of refraction of about 1.5, however, which represents the index of refraction in the protein itself, you find that the double refraction of flow disappears entirely. This indicates that this double refraction of flow you have been observing is due solely to the orientation of these rod-like particles in the flowing stream. I know you must be familiar with the way this rod-like material behaves in a flowing solution because you have seen logs in your rivers here and they give the same type of orientation in a flowing river.

Now you can make some practical use of this particular property of tobacco mosaic virus. It has nothing to do with the virus activity, and is simply caused by its unusual shape. You can determine not only the intensity but the direction of flow from studies such as this at every position around an object which is moving through the solution. I suppose you can visualize immediately the potential uses so far as the Navy is concerned for a solution of this particular type. I envisioned such possibilities quite early and put away two or three tons of diseased Turkish tobacco plants, expecting that I would get a call for material to be used in model experiments such as this. Eventually interest was shown and we got together about a pound of the purified material and sent it out for tests in model towing tanks. It turned out that this purified tobacco mosaic virus was by far the best of any material which had ever been used in their experimental tank experiments for determining the shapes of torpedoes, pontoons, and undersea craft. This is just a little sidelight on some of the potentialities of certain of the viruses. I should repeat that it has to do solely with the shape and with nothing else in connection with this particular virus.

If you examine solutions of tobacco mosaic virus by means of the electron microscope and measure up these particles which represent the rod-like individual particles of tobacco mosaic virus, you find that they are about 15 millimicrons in width and 300 millimicrons in length. Actually about 70 per cent of all the rods present are about 300 millimicrons in length. Other recent experiments indicate that from the standpoint of its length this is actually the most homogeneous material that has ever been found.

Studies have been made of the relative amounts of tobacco mosaic virus which are present in various hosts. Tobacco mosaic exists in extraordinarily high concentrations in the Turkish tobacco plants, somewhat less in tomato, and still less in the spinach plant. It is interesting that tobacco mosaic exists in different amounts in different hosts and that other viruses, like bushy stunt virus, etc., exist in still smaller amounts.

This is an important principle if you are interested in isolating and purifying a virus. If you make such studies, you must be sure that you harvest your host plant at the correct time after inoculation. In the case of alfalfa mosaic virus in Turkish tobacco the maximum amount of virus is reached about 12 days after inoculation. As time goes on, the amount of virus drops off very rapidly. It is obvious that you must be acquainted with the time at which it reaches its maximum; otherwise, if you attempt to harvest the virus, you may miss it entirely.

Electron micrographs of a purified preparation of a tomato bushy stunt virus impress one that all these viruses, plant viruses particularly, are not rod-shaped entities. The molecules of bushy stunt virus are small spheres, about 40 millimicrons in diameter. An interesting observation is that this particular virus can be obtained in the form of crystals.

I had expected to go on and discuss the problem of mutation, but I see that the hour of eleven has arrived. This general question of mutation and the nature of the changes in the chemical structure which takes place when the virus mutates will fit quite well into the talk this evening. With your permission I shall stop now and add the last ten minutes or so to the talk tonight.

## QUESTIONS AND ANSWERS

Question: I have a question about the individual cells. In the case of tobacco mosaic, they appear to be in great bundles, side to side.

Dr. Stanley: I don't know the significance of your

question. Are you thinking of the reproduction process —is that what you have in mind?

Question: Or a conjugation process?

Dr. Stanley: All I can tell you is that is a question about which we know nothing. In the case of both plant and animal viruses we do not know whether the substance of the inoculum ever gets into the progeny. In other words, we do not know whether they divide after the manner of ordinary bacteria and other organisms or whether the reproductive process involves some new type of process—some cataclysmic event—a process in which a new structure suddenly appears alongside the particles of the inoculum. That is one of the very important problems in the virus field.

# The Effects of Plant Viruses on Their Hosts

J. A. MILBRATH

THE effect produced by plant viruses on their host plants has been of great importance to virus research. Viruses have been impossible to culture or reproduce outside of the living cells, and without the electron microscope they would be impossible to detect unless they caused some abnormal condition in their host.

A very definite relationship exists between viruses and their host plants. Some viruses are so specific that they are known to infect only a single species, while others are able to infect plants from many different genera or even different families. Varieties of the same species often vary greatly in their response to a given virus. Some varieties are immune, some are symptomless carriers, while others may respond with a lethal reaction. Some plants are so intolerant of certain viruses that a severe lethal reaction occurs at the point of entry of the virus particle into the plant, resulting in an area of dead cells through which the virus is unable to move. The virus remains confined to these areas which are called local lesions.

Environment also has a marked effect on symptom expression. At one temperature a virus symptom may be completely masked, at another, show as a mild mosaic mottle, and still at a different temperature be expressed as a severe necrosis. The intensity of light, the vigor of the plant, the relative humidity also affect symptom expression.

In general a specific virus will affect a plant in a manner so characteristic that it can be readily recognized. However, there are some unrelated viruses which may cause identical symptoms in a given host. When considering all these features of host-virus relationships, it is not surprising to find considerable confusion in virus literature. Since the naming of viruses has been based primarily on symptom expression on a given host, it is not uncommon to find 20 or more names for the same virus.

Many terms descriptive of virus expression frequently are found in virus names and in literature on virus diseases. Such terms as mottles, mosaics, necrosis, yellows, dwarfs, stunt, leaf roll, ringspots indicate the effects commonly observed in virus host reactions. A brief discussion follows illustrating these terms and other virus behaviors.

### MOTTLES AND MOSAICS

Viruses frequently injure the chloroplasts and destroy much of the chlorophyl to give the foliage a chlorotic appearance. Often this chlorosis occurs unevenly in irregular patches of cells and causes a mottled appearance which frequently is called mosaic. With some viruses the pigment is only slightly reduced in these areas and the mottles are made up of light and dark green patterns. In others nearly all of the pigment is destroyed to give yellow or white patches scattered with the normal green. With some diseases the pigment is first destroyed along the veins and the interveinal areas remain green, while the reverse reaction occurs with other diseases. The chlorotic areas may form definite patterns in the form of concentric ringspots, bordered rings or bordered bands which zigzag through the leaf tissue forming various shaped patterns.

### NECROSIS AND LOCAL LESIONS

Many host tissues are so intolerant of the virus reaction that the cells die soon after invasion. This cell necrosis may be limited to the entry point of the virus on the inoculated leaf, or it may occur as a phloem necrosis, stem streaking, vein burning or as necrotic leaf spots. Many viruses develop only the local lesions on at least one of their host plants. Several layers of host cells around the point of entry of the virus particle may be killed. In some instances this reaction is so rapid that the cells die before the virus can move to the adjacent cells and the virus remains inactive in the local lesion. Other viruses

may first cause a local necrotic lesion, and then move out into other tissues where it may appear as faint mottles or may cause severe necrosis. Since the terminal meristem in the stem is one of the most active tissues it may rapidly become necrotic and the stem die back for several inches. The necrosis may be confined to the epidermal cells or even to the leaf hairs.

### YELLOWS GROUP OF VIRUSES

Many virus diseases belong to the yellows group. Here the plants react in a systemic manner and become uniformly chlorotic. These diseases are characteristically vascular in nature and never produce the characteristic mottles or necrotic spotting on any of their host plants. These viruses commonly produce a phloem necrosis and the transport of food materials is greatly disturbed. As a result affected plants are often dwarfed and misshapen, and excessive twisting or branching may occur.

### DWARFING AND STUNTING

Most plants affected with any virus show some reduction in size; however, some viruses produce an extreme dwarfing or stunting on their host plant. A plant that would normally grow two to three feet tall may be dwarfed to six inches by a virus. The plants may or may not show other foliage symptoms such as mottles or yellows. Some of these dwarfed plants actually appear darker green than normal. A weak, spindly type of growth is characteristic of some virus reactions.

### MALFORMATIONS AND DISTORTIONS

Witches' Brooms and dense rosettes of abnormal growths may be formed by the stimulation of excessive branching or axilary bud development or by the shortening of the internodes. The viruses causing such reactions usually are similar in nature to the yellows type. Leaves are often misshapen and malformed. Many of the mosaic types of viruses cause a puckering or rugosity of certain leaf areas, or may prevent parts of the leaf from expanding normally. Simple leaves may be reduced to strap-like structures, and compound leaves may be so devoid of expanded parenchmatous tissue that they appear like fern leaves. Veins may become distorted, swollen, or cleared of pigment. Leaves may become so twisted and malformed that they bear no resemblance to the original leaf. Flower parts may become enlarged or revert back to leaf-like structures. Roots may develop swollen areas or tumors. On trees various types of knots, cankers, and rough bark conditions frequently develop.

### EFFECT ON FRUIT DEVELOPMENT

Viruses may cause abnormal fruit development. In many cases this is brought about by disturbed food transport or low vigor of the affected plant. Some viruses produce specific reactions on the fruits without producing apparent symptoms on other plant parts. Peach Wart is an excellent example of this type of disorder. The foliage and growth of the tree appears normal, but as the fruit forms, a ring of stony wart-like structures develops about the stylar end of the fruit. Cherries are affected with several strains of similar viruses which cause the fruits to remain small, colorless and without flavor.

### LATENT VIRUSES OR SYMPTOMLESS CARRIERS

Many plants may act as carriers of a virus without expressing any visual symptoms. The potato is a classical example of a host with this type of virus. Most of the commercial varieties of American potato are so uniformly infected with a virus, that the juice of any tuber selected, when rubbed on a healthy tobacco or tomato plant, will produce visible evidence of a virus. Although a plant may express no visual symptoms, yield and growth studies indicate that a symptomless carrier does not grow or yield as well as a virus-free plant of the same variety.

### VIRUS STRAINS

There is considerable evidence that many viruses mutate or change in their chemical make-up to give rise to new forms or strains. A new strain usually resembles the mother virus in most properties, but will show some deviation from the original type. Quite often this is in the degree of severity of symptom expression. Tobacco mosaic, because it has been studied more than any other virus, has been shown to undergo frequent changes. Over 50 different strains of tobacco mosaic strains are now known to exist.

### EFFECT OF HOST ON SYMPTOM EXPRESSION

The same virus may infect a great variety of host plants and the type of symptom expressed on these different plants may vary greatly. The same virus may show no effects on one host, cause a mild mottle on another, local lesions on another and still be so lethal on still another host that the plant is killed within a short time after infection. In the same manner two unrelated viruses may cause symptom expressions on the same host so similar that it is impossible to detect which of the two viruses is present. Two or more viruses may infect the same plant at the same time. Each virus may express a symptom picture

independent of the other, or the two may unite to give a reaction very much different from that produced by either virus alone. Combination streak of tomato is a well-known disease which illustrates this behavior. Tobacco mosaic virus when inoculated into tomato produces a prominent mottle but does not greatly affect the growth of the plant. The healthy potato virus (Potato Virus X) when inoculated into a tomato plant also produces a mild mottle, some chlorosis and some necrosis depending on the severity of the strain present. When these two viruses are simultaneously inoculated into a tomato plant, small plants are killed with a rapidly spreading necrosis. Larger plants develop a necrotic leaf spotting, necrotic streaks develop on the stems, and frequently the terminals are killed. The fruits become marked with greasy necrotic spots and are misshapen.

The break of the color pattern of tulips has been shown to be caused by the interaction of two viruses, one which tends to remove color and the other to increase or deepen the color. When both viruses are present the color pattern is determined by whichever virus predominates in concentration.

SUMMARY

From this discussion it can be seen that the virus and its host have a very close relationship. It is impossible to say whether the host or the virus is responsible for the symptom expressed. Perhaps it is the joint response.

# Neoplasms Induced in Plants by Viruses

FRANK P. MCWHORTER

A NEOPLASM is an uncontrolled new growth of tissue. Perhaps for the botanists present, I should compare the term "neoplasm" with the term "hyperplasia." The terms have somewhat the same meaning, but "neoplasm" relates more specifically to the continued proliferation of the affected tissue or group of cells. Neoplasms are the precursors or, more literally speaking, the progenitors of cancer. When supporting organisms lose all control of the affected part and the cells composing the localized neoplasm become malignant and invasive, the result is cancer.

There are at least four distinct virus diseases of animals that are characterized pathologically as malignant neoplasms. Thus, there are four cancer diseases of animals caused by viruses. Viruses have not as yet been proved to be the specific cause of any cancer disease of man. Today, we will discuss how closely neoplasms in plants approach the status of true cancer.

In plants, the best development of a neoplasm and the closest approach to malignancy are attained by crown gall—a bacterial disease. That is proved by the former excellent cytological investigations of Michael Levine and the recent physiological studies of Armin C. Braun. Braun has shown that the secondary tumors of crown gall may contain no bacteria and that such tumors are capable of autonomous development when grown in tissue cultures. These neoplasms in tissue culture, when implanted into healthy plants, proceed to develop typical crown galls using their own cells in the process. This performance is a close approach to typical animal cancer where no causal organism is known. The other neoplastic diseases of plants of immediate interest are caused by viruses.

To appreciate virus neoplasms in plants, we must consider the principal effects of virus on cells. These are:

| | |
|---|---|
| Necrotic | Hyperplastic (Neoplastic) |
| | Hypertrophic |
| | Hypoplastic |

The pathological anatomy of virus diseases proves that most viruses induce all these symptoms to some degree. In a few cases, the principal effect is hyperplasia and proliferation. When hyperplasia is the principal and continued effect on the cells of the plant, neoplasms may result and become apparent as an external symptom.

The external symptoms in plants that are of special interest because of their cancer-like attributes are:

a. Galls or tumors

b. Enations or intumescences

The development of these symptoms from infection by viruses is comparatively rare. Enations do occasionally occur in a few virus diseases, where they are not the principal symptoms. In the case of enation mosaic of peas, they are the principal symptom. We will first discuss plant virus tumors; then enations. There is only one plant tumor disease known to be caused by a

virus. That disease is the wound-tumor disease discovered by L. M. Black.

The wound-tumor disease is caused by a virus that is not mechanically transmissible. It is carried by and reproduced in Agallian leafhoppers. These insects are able to transmit the virus to many kinds of plants, especially legumes. Tumors are regularly produced on the roots of its hosts. The performance of the virus in sweet clover is especially interesting. If an infected sweet clover plant is allowed to grow normally, no tumor will form on the stems. If the stem is punctured with a needle, the odds are three to two that a tumor will form at the point of injury. This, then, is a case where a mechanical injury plus a virus produces a tumor. These tumors are composed of abnormal cells and can be grown in tissue cultures. When so grown, they continue to form anaplastic masses incapable of forming roots, stems, or leaves. Individual cells frequently contain round basophylic inclusions. This last point is mentioned because these inclusions are not unlike some reported in cancer cells in animals.

Enation mosaic of peas is the plant virus disease that develops true neoplasms in addition to various hyperplasias that are quite commonplace symptoms of plant viruses. The neoplasms associated with the tumor virus all arise from one tissue only, namely: the pericycle tissue. The neoplasms and abnormal growths, characteristic of the enation mosaic of peas, can and do arise from any tissue in the foliage of the plants. In fact, the most striking anatomical feature of this disease is the ability of the virus to control a single tissue within the plant and cause that tissue to grow into a histological monstrosity. Such selective tissue transformation is typical of animal cancer.

Invasiveness is an important criterion of whether a neoplasm is approaching malignancy. We will illustrate the pattern of invasive plant cells with the mechanism of dodder where true invasion is attained.

Lantern slides were shown of leaves of *Vicia faba* and *Pisum sativum* to illustrate the tissue and cell changes that have the form and structure of neoplasms. Appropriate controls and the virus history of the plants prove these neoplasms are truly of virus origin. The most profound changes are found in the pod of the pea, *Pisum sativum*. There, extreme chromosome modifications occur. Cell gigantism and multinucleate cells are of common occurrence.

The neoplastic masses originate usually from the outer bundle structures of veins. Often, the terminal cells of these masses appear invasive. The significance of this circumstance was shown by photographs of cells of dodder invading a host plant. Such dodder cells illustrate the morphology of plant cells where the invasive tendency is the principal property. The similarity between the boundary cells of the neoplasms and these dodder cells was pointed out. A close approach to invasiveness does occur, but metastasis, as in animals, is not evidenced.

Thus, evidence is presented by photographs that the virus of enation mosaic can produce true neoplasms in its hosts; that while there is not evidence of metastasis, there is good evidence for invasive tendencies. The cells of these neoplasms are always anaplastic and totally unlike their parent cells. This circumstance and the regular development of abnormal mitoses are characteristic of a malignant neoplasm.

Thus, the enation legume disease should be a good subject for physiological study of a true virus disease characterized by neoplastic growths that have many aspects of cancer. And this virus disease can be transmitted mechanically, thus making it readily adaptable to greenhouse cultures and subsequent laboratory techniques.

# Viruses and Genes

ERNST J. DORNFELD

VIRUSES were discovered just a few years before the reestablishment of the Mendelian Laws by Correns, Tschermak, and deVries in 1900. Yet during at least 35 years of genetic research they offered little interest to students of heredity. One will not find them mentioned in genetic textbooks of ten years ago, except possibly for some passing comment or conjecture on the origin of life. Today this situation has changed completely. In the course of a few recent years viruses have taken on a new importance. Every student of genetics or cellular biology, even though his own experience has never included direct study of viruses, is keenly interested in these bodies. As has happened many times before in the progress of science, we see once more an instance in which separate lines of advance, this time genetics and virology, have

come together and established fruitful association. The chemical discoveries of Dr. Stanley, it may be said at once, have been a leading factor in hastening this new relationship.

In order to understand the geneticist's interest in viruses it is necessary to retrace a little history. The developments are tied up in part with the growth of genetical theory.

In the years following the rediscovery of the Mendelian laws, attention was focused mainly on the relationship of genes to chromosomes. The demonstration of this relationship was accomplished with brilliant success, culminating with the production by Morgan and his group of maps showing the linear arrangement and exact positions of the genes on the chromosomes. Such studies necessarily made use of sexually reproducing organisms that displayed heritable variabilities, or mutations. The construction of maps became possible in principle for all plants and animals whose chromosomes are shuffled and exchanged in meiosis and syngamy. Blue-green algae and bacteria, which were thought to lack a nucleus, not to mention chromosomes, were left out of the picture. They represented a genetic vacuum.

The pattern of the transmission of characters from generation to generation and its cytological explanation constituted for some time the principal content of genetic study. Meanwhile, however, interest in the fundamental nature of the chromosomal gene was growing. The discovery by H. J. Muller, in 1927, that X-rays were effective in inducing mutations, greatly spurred this on. During this same period it happened that biochemists were reinvestigating the constitution of nucleoproteins, known since the previous century to be characteristic substances of cell nuclei. It became possible to apply their knowledge and methods eventually to the analysis of isolated nuclei and even chromosomes. Without relating any of the interesting details of this important achievement, suffice it to say that the chromosomes were shown to consist chemically of highly polymerized nucleo-proteins, the nucleic acids being principally of the desoxyribose type. Genes themselves have not, thus far, been isolated, but their specificity lies, apparently, in the permutations and combinations possible within the gigantic nucleo-protein molecules.

Biologically, the fundamental properties of genes, it will be recalled, are twofold. The first, upon which the basic phenomena of heredity rest, is that of self-reproduction carried on without change (except for rare mutations) through millions of cell generations. The second, of foremost importance to the individual organism, is the control, by genes, of differentiation, or development. This is basically a chemical process, the phenotype being the final outcome of chains of chemical reactions whose patterns are hereditarily determined. How the genes are involved in this process is one of the principal concerns of present-day genetics. It is in this field of enquiry that the viruses were first forced onto the attention of geneticists.

Cellular differentiation is itself localized in the cytoplasm. Structural proteins are synthesized and accumulated outside of the nucleus, but in the absence of the nucleus this activity does not continue. Its cessation is not, however, abrupt. It has long been known, for example, that when enucleated egg fragments of echinoderms are fertilized by sperm of a foreign species, the early stages of development are maternal, the reciprocal cross behaving in the same manner. There is, thus, a temporary continuation of gene-like action, even though the chromosomes concerned are absent. At the same time, the action of the paternal genes lags, though it takes full effect after a while. Several interpretations of this phenomenon are possible, but one of them postulates that gene-like substances exist in the cytoplasm, where in the presence of raw materials they carry on their specific synthetic processes. These so-called plasmagenes are capable of self-reproduction, often only in the presence of specific nuclear genes, and are cytoplasmically transmitted during cell division. They have, thus, a certain degree of autonomy. When nuclear support is lost (as by absence of chromosomal genes or mutation), the plasmagenes may not be reformed and the existing supply becomes dissipated in successive cell generations. At this point gene action appears to cease.

In recent years several cases of plasmagene action have received intensive study. One of these, investigated by Dr. T. M. Sonneborn, concerns the ciliate protozoan, *Paramecium aurelia.* It will serve well to illustrate some of the main features of the phenomenon.

Certain strains of *Paramecium* produce and liberate a substance, "paramecin," which kills other strains known as "sensitives." The traits of killers and sensitives are inherited. However, when appropriate crosses are made, the killer trait turns out to be cytoplasmically inherited, irrespective of the nuclear constitution. The cytoplasmic factor, or plasmagene, determining paramecin production is known as "kappa." When the

genes of a killer strain are introduced into a sensitive animal through conjugation, but without transference of cytoplasm, no paramecin is produced. The kappa plasmagene therefore never arises in the absence of preexisting kappa, and, unlike some other cases of cytoplasmic inheritance, the nuclear genes do not start its production. A certain nuclear gene, K, is however, necessary for its maintenance. Kappa will disappear in the absence of K, but the reproduction of the chromosomal K is entirely independent of kappa, and, as already pointed out, it cannot produce kappa when the plasmagene is absent. The extensive studies of Dr. Sonneborn have brought to light further important points, which may be briefly summarized as follows: (1) The plasmagene kappa mutates; (2) the rate of multiplication of kappa has been established and is independent of the rate of cell division; (3) random distribution of kappa particles accompanies cell division; (4) high temperatures and X-rays destroy kappa; (5) a single *Paramecium* contains between 200 and 1,000 kappa particles; (6) high concentrations of particles are needed to produce paramecin; (7) a single particle is sufficient to produce more; (8) the kappa particles are visible: they are from 0.2 to 0.8 micron long; (9) they contain desoxyribonucleic acid; (10) the particles can enter a cell from the surroundings (broken-up bodies of killers) and proceed to multiply.

Quoting Dr. Sonneborn: "Kappa is like a gene in some respects, different in others. It resembles a gene in its determination of a hereditary unit, its self-duplication, its mutability, its chemical composition, and its 'dosage effect,' i.e., the dependence of effect on concentration in the cell. It differs from a gene in the usual number per cell, the mode of distribution at cell division, the occasional lack of synchronism between its rate of duplication and the rate of cell duplication, its high sensitivity to environmental conditions, its size, its cytoplasmic localization, and its capacity to enter a cell from the milieu and be maintained thereafter."

To students of viruses these properties sound amazingly familiar. The parallelisms need no emphasis. Kappa could be described as a virus as well as a plasmagene.

It would be profitable to review the investigations dealing with several other instances of cytoplasmic inheritance, but time does not allow this. They differ in various details but leave no doubt that cytoplasmic units do exist capable of self-duplication and having some degree of autonomy. With reference to the mechanism of cellular differentiation it has been suggested that genes always operate through the mediation of plasmagenes, but such a claim is premature. What the extent of such a relationship may be, however, is a challenging problem. All embryonic cells are known to be rich in cytoplasmic particulates carrying nucleic acids, mainly of the ribose type. Whether these have varying specificities and may be likened to plasmagenes is impossible to say. Dr. Sewall Wright has theorized that in a developing organism plasmagenes become systematically modified under the influence of local cytoplasmic conditions without losing their ability to multiply. An orderly sequence of such modifications might, in fact, be the basis of differentiation. Plasmagenes would thus complement the action of nuclear genes. Spiegelman and Kamen have extended this idea by assigning to the plasmagenes the cytoplasmic control of enzyme and protein synthesis. Competitive interaction among them would determine the enzymatic make-up of the cytoplasm. In this manner different cells would come to differ in their enzyme constitution, even though their nuclei carry the same genotype.

Returning to the question of virus relationship, Darlington has suggested that viruses may be plasmagenes in the wrong host, or plasmagenes made pathogenic by mutation. He bases this comparison on the similarities in chemical and physical properties, mutability, reproduction, action of two viral types in the same host, and the existence of symbiotic species indistinguishable in their host effect from plasmagenes. The case of the milk virus which transmits mammary cancer in mice is particularly challenging in the light of this comparison. Carcinogenetic agents, which are known to have mutagenic properties, might operate by inducing plasmagene mutations, thus producing particles which are similar to the virus in characteristics and effects. The relation between plasmagenes and viruses seems to be a reasonable one except for the appearance of some recent work which shows bacterial viruses to possess totally unexpected and amazing genetic complexity.

The bacteriophage of the colon bacillus (*Escherichia coli,* strain B) consists of tiny virus particles 45 to 100 millimicra in diameter. After penetration of the bacterial cell each particle produces from 100 to 300 replicates in the course of 15 to 30 minutes, the cell disintegrates, and the brood of infective particles is liberated.

This phage has various mutant forms, some of which alter the host range, others modify the

type of plaque produced by the lysis of bacteria on an agar plate. The spontaneous mutation frequency has been measured and has been found to vary, as in higher organisms, from 1 per 1,000 to 1 per 100,000,000 particle generations.

If two unrelated viruses infect a bacterial cell simultaneously, only one type survives, the other is lost. On the other hand, when two closely related mutant types, *a* and *b*, infect a single host an entirely different thing happens, as reported in 1946 by Delbrück and Bailey and by Hershey. Both *a* and *b* reappear in the progeny, and in addition to this, a third and fourth type are present. The third carries the characteristics of both *a* and *b*, and the fourth resembles the parent strain from which *a* and *b* were originally derived. This situation clearly suggests that each virus particle contains more than one hereditary unit and further, that these units are capable of recombination, much as in sexually reproducing organisms. Hershey and Rotman believe even to have found some evidence for linkage.

The genic complexity and powers of recombination in bacterial viruses were confirmed with even more striking data by Luria in 1947. Using ultraviolet radiation of 2537 A. to inactivate phage suspensions, he found that one quantum inactivated, or induced a lethal mutation in, one particle. By adjusting the dosage it could be arranged that most particles absorbed several quanta each. Such a suspension was then diluted and mixed with bacteria to make a proportion of one inactivated particle per cell. Very few bacteria were destroyed, as was to be expected. However, when the mixture was arranged to provide two particles per cell, a high percentage of bacteria were lysed and these liberated viable phages. Reactivation had been accomplished. Presumably this could have happened only if the lethal mutations were not all of the same kind, and normal genes could be exchanged for lethal ones. Properly formulated experiments showed that between 15 and 50 different loci could be inactivated, or that that many genes exist in a single virus particle! The number varies with the absolute size of the particle, somewhat fewer than 8 or 10 occurring in small forms.

Using Luria's data on the T2 bacteriophage, indicating the presence of 25 genetic units, Zahler has recently published a calculation on the number of nucleotides per gene. Knowing the mass of the virus and the percentage phosphorus content, which appears to be contained almost entirely in the nucleic acid fraction, some of the genes, at least, would contain not more than 8,000 nucleotides. This corresponds to a molecular weight, for the nucleic acid portion of the genes, of less than 3 million.

The possibilities now opened by viruses for fundamental advances in genetics are tremendous. That viruses possess the kind of genetic complexity demonstrated by Hershey and Luria would have appeared incredible only five years ago. That they could be considered objects of genetic interest at all would have seemed unpromising before Dr. Stanley's discovery of their chemical nature. Geneticists have every reason to look forward to an exciting virological future.

# The Inhibition of Viral Multiplication

ARTHUR W. FRISCH and THOMAS HOSTY

THE introduction of sulfonamides and antibiotics into the therapeutic armamentarium has stimulated renewed interest in the treatment of viral and rickettsial diseases of man and animals. As recently as 10 years ago it was generally believed, and so stated, that the intracellular position of these parasites made therapy untenable since any agent which could reach the virus would also destroy the cell. The recognition of the competitive antagonism between the sulfonamide drugs and p-aminobenzoic acid (1, 2, 3) coupled with the fact that the virus of lymphogranuloma venereum was found to be susceptible to the action of the former (4, 5, 6) has made it necessary to abandon the above position. The aim of today's chemotherapy is to administer compounds which supposedly interfere with an enzyme or an essential metabolite of the parasite, the host, or both, thereby preventing multiplication of the infectious agent. Theoretically the damage to the parasite should be irreparable, whereas the injury to the host should be minimal. Within the past few years such a result has been achieved with a number of antibiotic agents.

In Table 1 the rickettsias and viruses have been grouped according to size and in relation to experimental and clinical results of treatment with a variety of chemotherapeutic substances. It should be emphasized that a number of the claims have not been properly verified. However, the

results are of extreme interest, in that those rickettsias causing typhus fever, spotted fever, tsutsugamushi disease, rickettsial pox, and Q fever have proved to be susceptible to the *in vitro* and *in vivo* action of aureomycin and chloroamphenicol. In general it may be stated that those viruses which range in size from 400 to 450 mμ are inhibited by most therapeutic agents, whereas the effect is less pronounced and more selective when the smaller ones are tested. The favorable clinical results claimed for chloroamphenicol with herpes simplex, varicella, and mumps viruses require confirmation. If one considers that immune serum was the only form of treatment available just 12 years ago, the progress made in the treatment of viral and rickettsial diseases is amazing, indeed.

Unfortunately the mode of action of many of these drugs is poorly understood. The information available at present is given in outline form below:

SULFONAMIDES (1,2,3,7)

1. Antagonist to p-aminobenzoic acid which is a precursor of folic (PGA) acid, an essential growth factor.

PENICILLIN (8,9,10,11)

1. Prevents transportation of glutamic acid across the cell membrane of gram positive organisms.
2. Interferes with dephosphorylation of mononucleotides.
3. Prevents protein synthesis.
4. Prevents peptide synthesis.
5. Inactivates dehydrogenases.

STREPTOMYCIN (12,13,14,15)

1. Precipitates some nucleic acids.
2. Blocks conversion of pyruvate to acetic acid.
3. Lipositol inactivates streptomycin; both are inositol derivatives.

CHLOROAMPHENICOL (16)

1. Inhibits horse liver and bacterial esterase, the only enzymes affected out of 45 tested.
2. May, therefore, inhibit hydrolysis of fat or utilization of organic acids and alcohols.
3. It should be noted that most animal viruses contain lipids.

AUREOMYCIN AND TERRAMYCIN

1. Mode of action unknown.

As can be seen from these summary data, there is much to be learned about the mechanism of action of most of the antibiotic agents. Our information about the sulfonamides rests upon the most secure foundation, but even here the evidence has been questioned (7, 18). The studies of Smith, Worrel, and Swanson (16) on the esterase inhibiting activity of chloroamphenicol are of particular significance because of the fact that 45 other enzyme systems known to occur in *E. coli* were not affected by this drug. They observed at least three definite zones of response with varying concentrations of chloroamphenicol. Small doses produced no effect; increasing concentrations showed primary inhibition of esterase activity followed by a zone of marked acceleration, and finally contact with 3 to 50 micrograms resulted in a sharp decrease. The antiesterase activity was incomplete when cells or mitochondria were used, suggesting that there are at least two barriers in the host cell which might prevent reaction on tissue enzymes. If one considers the wide range of action of chloroamphenicol against gram positive and gram negative bacteria, rickettsias, and viruses, it seems likely that a study of lipid metabolism may furnish a fundamental clue to the chemotherapy of virus diseases. A warning has been issued against prolonged use of

Table 1. EFFECT OF ANTIBIOTICS ON RICKETTSIAS AND VIRUSES

| Disease agents | Size mμ | Sulfonamides | Penicillin | Streptomycin | Chloroamphenicol | Aureomycin | Terramycin |
|---|---|---|---|---|---|---|---|
| Rickettsias | 475 | — — | — — | +? — | + + | + + | + + |
| Psittacosis | 450 | + + | + + | — — | + + | + + | + 0 |
| Lymphogranuloma | 440 | + + | +? — | — 0 | + + | + + | + 0 |
| Trachoma | | 0 + | 0 +? | 0 — | 0 + | 0 ± | 0 0 |
| Atypical Pneumonia | | 0 — | 0 — | 0 — | 0 + | 0 + | 0 +? |
| Mumps | 340 | — — | — — | — — | — + | — — | 0 0 |
| Vaccinia | 225 | — — | — — | — — | — 0 | — 0 | 0 0 |
| Varicella | 200 | 0 — | 0 — | 0 — | 0 +? | 0 — | 0 0 |
| Herpes Zoster | 200 | 0 — | 0 — | 0 — | 0 +? | 0 + | 0 +? |
| Herpes Simplex | 150 | — — | — — | — — | 0 +? | 0 ± | 0 0 |
| Influenza | 115 | — — | — — | — — | — — | — — | +? — |
| Kerato Conjunctivitis | 85 | 0 — | 0 — | 0 — | 0 +? | 0 + | 0 0 |
| Equine Encephalomyelitis | 50 | — — | — — | — — | — — | — — | 0 0 |
| Poliomyelitis | 25 | — — | — — | — — | — ± | — — | 0 0 |

First row = Experimental. Second row = Clinical. 0 = Not done. — = Ineffective. + = Effective.

both aureomycin and chloroamphenicol by the finding that 100 gamma per ml markedly inhibits proliferation of fibroblasts and epithelial cells in tissue culture (25).

The rickettsial agents of disease are usually classed with the viruses, but in many ways they can be considered as smaller counterparts of bacteria. Most rickettsias multiply within the cytoplasm of the host cells with the exception of *R. rickettsiae* which prefers the nucleus. In this respect they are not so different from the malarial parasites or such bacteria as *P. tularensis* and *B. abortus* which also prefer an intracellular environment. *C. burneti*, the causative agent of Q fever, is the only rickettsia which is classed as filterable. Therefore, the property of multiplication in the presence of living cells is the one feature common to rickettsias and viruses.

Figure 1 lists the chemical and antibiotic agents which influence the multiplication of rickettsias. It can be seen that the benzene ring, nitrogen, and frequently chlorine are necessary for antirickettsial activity. To date, the most effective agents for the treatment of human disease have been chloroamphenicol and aureomycin. The use of p-aminobenzoic acid in the treatment of rickettsial diseases was first suggested by the fact that sulfonamide derivatives were ineffective and even harmful (18, 19). These parasites, then, are unique in that they resent the presence of folic acid, an essential growth factor for most bacteria, and thrive best when its synthesis is blocked.

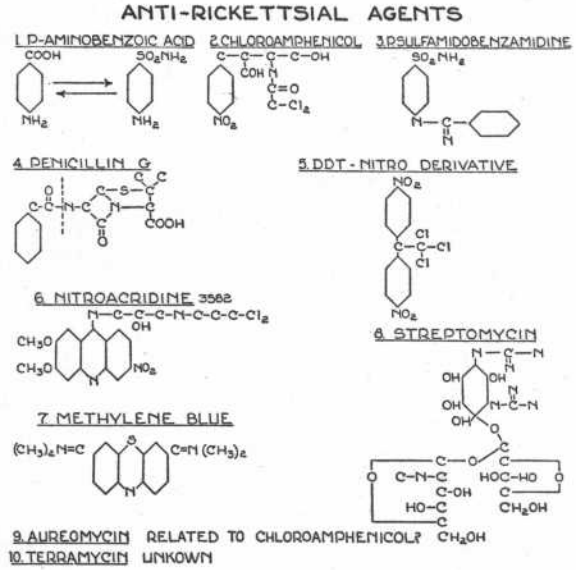


*Figure 1.*

The psittacosis-lymphogranuloma group have been studied most intensively from the point of view of *in vivo* inhibition. These viruses are characterized by Findlay (20) as follows:

1. Large size: 200-400 mμ.
2. Stain readily.
3. Spherical or hemispherical with limiting membrane.
4. Morphologic sequence of multiplication.
5. Contain thymonucleic acid (DNA).
6. Give cross complement fixation reactions.

Included among the members are lymphogranuloma venereum, psittacosis, ornithosis, trachoma, inclusion conjunctivitis, mouse pneumonitis, cat pneumonitis, and meningo-pneumonitis viruses. The chart (Figure 2) indicates that a variety of chemical and antibiotic agents are capable of inhibiting the multiplication of the P-L group. Eaton and coworkers (21, 22, 23) have been particularly interested in the nitroacridine, nitrobenzene, and the arsenobenzamide compounds. Some of these bear structural relationships to chloroamphenicol and show moderate activity in eggs and mice against the P-L viruses. Morgan (24) has utilized the sensitivity of psittacosis virus to sulfadiazine as a tool for the study of the inactivating effect of PABA (p-aminobenzoic acid). He has found, in chick embryos, that PABA acts as a competitive inhibitor for sulfadiazine. Pteroylglutamic acid replaces PABA but this action is not reversed by sulfadiazine. Pteroic acid is also effective but glutamic acid is without activity. It is assumed from these data that sulfonamide interferes with the metabolism of PABA by the psittacosis virus which, unlike the rickettsias, requires folic acid as an essential growth factor. The fact that 4-aminopteroylglutamic acid (ami-

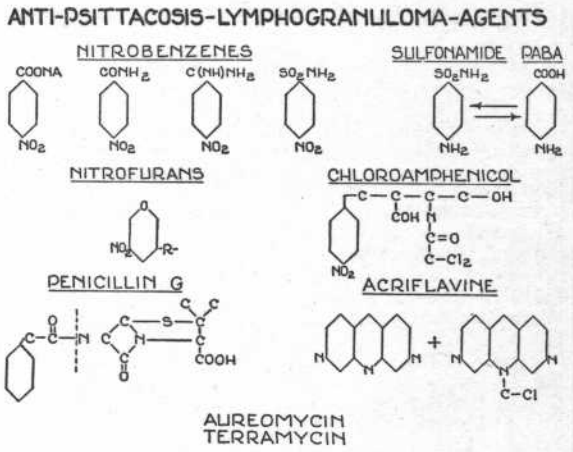


*Figure 2.*

nopterin) and other folic acid antagonists do not inhibit the growth of psittacosis virus is opposed to such a concept.

Among the group classed as the pox viruses are usually included variola, varicella, herpes zoster, vaccinia, and a number of agents producing pox-like diseases in animals. The infectious units are visualized as brick-shaped structures varying in size from 200 to 250 mμ and one member of the group, vaccinia, is known to contain at least three distinctly different antigenic components. Vaccinia virus has been a favorite tool for the study of chemotherapeutic agents (26, 27, 28, 29). However, relatively few have been found which are effective. Penicillin has been studied most intensively and it was observed that an impurity in the crude preparation, o-hydroxy-phenylacetic acid, is probably responsible for the antiviral activity (30, 31, 32). The observations on mercurochrome and quinine deal with fowl pox infections in chicks but lack confirmation.

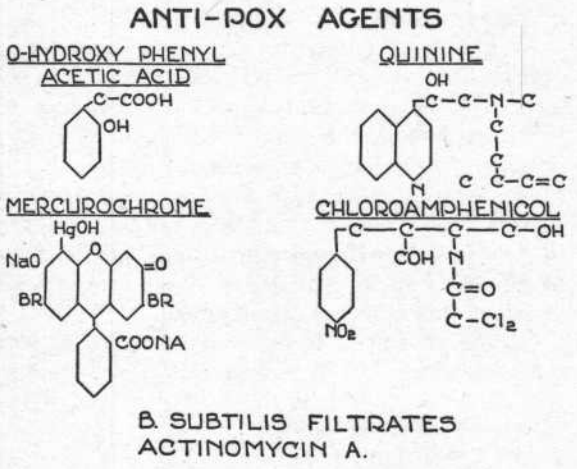


*Figure 3.*

The fact that the influenzal group of viruses grow readily in the chorioallantoic sac of the developing chick embryo, and that these viruses have the property of agglutinating red blood cells makes them useful as experimental agents for chemotherapeutic studies. A large variety of compounds have been tested for *in vivo* anti-influenzal activity without success (33, 34, 35, 36). Smadel *et al.* (37) observed that nitroacridine 3582 inhibited the multiplication of influenza B virus. These studies were extended by Green (38) and Rasmussen (39) and can be characterized as promising. Claims for the efficacy of atropine sulphate (40), the beta lactones (41) and tannic acid (42) have not been verified.

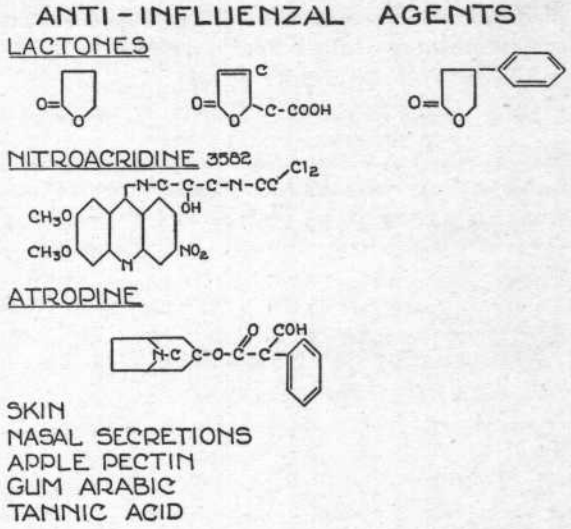


*Figure 4.*

For the past two years our laboratory has been interested in the effect of antimetabolites and enzyme inhibitors on the multiplication of influenza viruses. A number of chemicals are known which either function as metabolic antagonists or which have the property of inactivating either single enzymes or groups of enzymes both *in vitro* and *in vivo*. For example, malonic acid competes with succinic acid for the enzyme, succinic dehydrogenase. Potassium cyanide destroys cellular respiration by inactivating cytochrome oxidase. In the experiments to be presented, the inhibitors were first tested for their lethal dose in 10-day-old chick embryos. The amount used was that dose which killed 50 per cent of the embryos within 24 to 48 hours. A second group of eggs received half of the LD/50 dose and a third was given a hundredth of this amount. At the same time the embryos received 10 to 100 infecting doses of influenza A (PR8) or influenza B (Lee) virus. After incubation at 37° C. for a period sufficient to reproduce virus (24 to 48 hours), all dead eggs were discarded and the allantoic fluids were removed from the remaining eggs and pooled. Red blood cell hemagglutination titres and chick embryo infectivity titres were performed. Control eggs which had received an equivalent amount of saline and virus were included in each experiment. The results in Table 2 indicate clearly that in the concentrations used, none of the chemicals significantly reduced the ability of the influenza A and B viruses to multiply on the chorioallantois. Nevertheless, when one considers the mechanism of action of

these inhibitors, the data throw light on the mode of reproduction of the influenza viruses.

Table 2. EFFECT OF INHIBITORS ON THE MULTIPLICATION OF INFLUENZA A AND B VIRUSES

| | Influenza A (PR8) | | | Influenza B (Lee) | | |
|---|---|---|---|---|---|---|
| Mg per egg | LE | RCAT | IT | LE | RCAT | IT |
| *Sodium azide* | | | | | | |
| 0.26 | 19 | 5,120 | 7 | 18 | 80 | 6 |
| 0.13 | 12 | 1,280 | 7 | 8 | 160 | 6 |
| 0.0013 | 12 | 1,280 | 7 | 10 | 80 | 6 |
| ——— | 12 | 1,280 | 7 | 7 | 40 | 6 |
| *Nitrogen mustard* | | | | | | |
| 0.20 | 15 | 5,120 | 7 | 19 | 320 | 6 |
| 0.10 | 12 | 5,120 | 7 | 12 | 160 | 6 |
| 0.001 | 12 | 5,120 | 7 | 12 | 160 | 6 |
| ——— | 12 | 5,120 | 7 | 12 | 160 | 6 |
| *Sodium fluoroacetate* | | | | | | |
| 0.35 | 17 | 640 | 7 | 16 | 1,280 | 6 |
| 0.175 | 10 | 640 | 7 | 10 | 2,560 | 6 |
| 0.00175 | 12 | 1,280 | 7 | 12 | 2,560 | 6 |
| ——— | 12 | 1,280 | 7 | 12 | 2,560 | 6 |
| *3-Acetylpyridine* | | | | | | |
| 0.0006 | 24 | 640 | 7 | 24 | 640 | 6 |
| 0.0003 | 12 | 640 | 7 | 12 | 2,560 | 6 |
| 0.000003 | 12 | 640 | 7 | 12 | 2,560 | 6 |
| ——— | 12 | 640 | 7 | 12 | 2,560 | 6 |
| *Aminopterin* | | | | | | |
| 0.25 | 24 | 640 | 7 | 21 | 40 | 6 |
| 0.125 | 12 | 320 | 7 | 11 | 80 | 6 |
| 0.00125 | 12 | 640 | 7 | 11 | 80 | 6 |
| ——— | 12 | 320 | 7 | 11 | 160 | 6 |
| *Sodium citrate* | | | | | | |
| 16.6 | 24 | 5,120 | 7 | 24 | 80 | 6 |
| 8.3 | 12 | 5,120 | 7 | 9 | 80 | 6 |
| 0.083 | 12 | 5,120 | 7 | 12 | 80 | 6 |
| ——— | 12 | 5,120 | 7 | 7 | 40 | 6 |

LE = Live embryos. RCAT = Red cell agglutination titre. IT = Infectivity titre.

## PROBABLE MODE OF ACTION OF INHIBITORS

SODIUM AZIDE (43, 44, 45)

1. Blocks cytochrome oxidase and catalase.
2. Blocks ATP formation.
3. Over-all effect is to stop cellular respiration and to slow the energy cycle.

NITROGEN MUSTARD (46, 47)

1. Combines with nucleic acid and arrests mitosis.
2. Inactivates choline esterase.
3. Inhibits pyruvate, amino acid, and glucose metabolism.

FLUOROACETATE (48)

1. Blocks acetate utilization and removes energy sources required in protein, fat, and carbohydrate metabolism.
2. Inhibits the oxidation of fatty acids.

3-ACETYLPYRIDINE (49)

1. Nicotinic acid amide antagonist.
2. Interferes with coenzymes I and II in energy cycle.

AMINOPTERIN (50)

1. Folic acid antagonist.
2. General growth depressant.
3. Interferes with cell maturation.

SODIUM CITRATE

1. Inactivates enzymes dependent upon calcium and magnesium.
2. Excess citrate ion in Krebs cycle.

Time does not permit a presentation of miscellaneous agents which are known to inhibit the multiplication of viruses. Outstanding among these are studies referable to bacteriophage, the effect of polysaccharides on viral multiplication, the use of podophyllin against the wart virus, malononitrile against the Lansing poliomyelitis virus, and others. In fact, bacteriophage alone could easily consume the time allotted for the entire symposium.

We have attempted in this discussion to indicate the direction of one phase of research in the field of viral multiplication. One might inquire, then, just how do the data fit with present theories of virus formation as summarized below?

1. Viruses possess all the enzymes necessary for metabolic purposes and also for the synthesis of new protein within the host cell. This concept would class them, like bacteria, as autonomous parasites and is generally rejected.

2. At present it is believed that viruses contain only those enzymes necessary for reproductive purposes and that the host cell contributes the metabolic environment.

3. A few individuals consider that viruses do not possess enzymes at all, but rather that they reproduce by directing the formation of new protein from enzymes already present in the host.

All of these concepts imply that in some manner, through enzymes, new virus protein is formed in a step-wise synthetic process involving the construction of larger and larger units which finally emerge as mature virus. The gradual synthesis of proteins from amino acids would be an example of such a step-wise process.

Some of the data which have been presented may be interpreted as favoring the synthesis idea. Thus, chloroamphenicol, by interfering with esterase activity, appears to inhibit the reproductive capacity of viruses, rickettsias, and a wide variety of bacteria, as well. Such a finding, if true, would imply a step-wise reproductive process

common to all three groups of parasites. We have also seen examples of selective inhibition by related compounds, as in the case of the rickettsial agents where the substitution of a sulfonamide group in place of a carboxy radicle in the same position on the benzene ring reverses the action of the drug. Data of this type suggest a complex and variable reproductive process, or else a secondary effect on the primary pathway. The results using enzyme inhibitors and metabolic antagonists are not in agreement with the concept of step-wise synthesis of virus protein from smaller units within the cell environment. It is difficult to visualize a complete immunity of the infected cell toward such drastic interference with metabolic processes as was imposed by the experimental conditions adopted. The results could be criticized on the grounds that all cells were not equally affected, or that the chemicals did not penetrate into the region where the virus was being formed. Even if such reasoning were correct, at least a partial decrease in virus titre should have occurred and this was not evident from the data which were obtained. In the case of the influenza viruses one is tempted to reject the unit synthesis theory as unlikely and to consider another alternative, namely, that influenza virus protein is formed by direct conversion from mature cell protein, a process which would involve a minimal expenditure of energy and relatively few, if any, enzymes. Some evidence in favor of such a hypothesis can be obtained from the electron microscope, from studies of similarities and differences between virus and cell proteins, as well as from the use of inhibitor techniques of the type described.

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# The Problem of Bacteriophage in the Dairy Industry

P. R. Elliker

FERMENTATION of milk sugar, lactose, by lactic acid bacteria has been employed for hundreds of years in the production of a variety of dairy products such as cheeses and fermented milks. The formation of lactic acid in such products is essential for desired flavor, physical change, and preservation. Lack of sufficient acid development may result in an inferior or worthless product.

It now is an accepted fact that destruction of lactic acid bacteria by bacteriophage, a bacterial virus, represents one of the most important causes of insufficient acid development in manufactured dairy products. This is an industrial problem and has parallels in other industrial fermentations. Examples are bacteriophage (phage) lysis of: *Clostridium* species in the acetone-butanol fermentation; *Bacillus polymyxa* in production of 2,3-butanediol; and *Streptomyces griseus* in streptomycin production. The decentralized nature of the dairy industry and wide variety of lactic acid streptococci employed as starter cultures in dairy products greatly complicate the bacteriophage problem for the dairy plant operator. Added to this is the ubiquity of lactic acid streptococci in nature which may thus provide a vast reservoir of phage races capable of attacking the starter cultures employed in the dairy plant.

Recognition of phage destruction of lactic acid bacteria is not a recent development. Hadley and Dabney (9) in 1925 described phage lysis of *Streptococcus lactis*. Whitehead and Cox (32) in New Zealand in 1934 reported inhibition of Cheddar cheese starters by an agent introduced into the culture medium by aeration. They soon identified the agent as phage (33). Their observations were confirmed by investigators in France, Australia, United States, Canada, and England (1, 2, 16, 17, 20, 30). The problem appears to be universal in the dairy industry. It also appears to be related, to some extent, to the advent of pasteurization of milk for dairy products. Raw milk usually carries sufficient numbers of lactic acid (milk-souring bacteria) that may substitute for those added in the form of a cultivated starter culture in the event the latter are attacked by phage. However, if the natural lactic acid flora is removed from the milk by pasteurization, and the added starter bacteria are destroyed by phage, there will be partial or total failure of the lactic acid fermentation in the product.

## Dairy Products Affected by Bacteriophage

Phage has been reported most frequently in connection with Cheddar type cheese. In the manufacture of this product, partial inhibition of lactic acid production frequently is referred to as a "slow vat" and nearly complete inhibition as a "dead vat." In addition to disruption of the carefully timed operating schedule in the cheese plant, production of insufficient lactic acid also leads to undesirable fermentations with consequent abnormal fruity, rancid, and putrid flavors, and excessive gas formation. The low pH attained in a normal, vigorous lactic acid fermentation prevents growth or toxin formation by chance pathogens that might be present in the early stages of manufacture. Inhibition of the lactic acid bacteria removes that protective factor. This problem always represents a potential hazard in cheese such as Cheddar, because the milk and curd are held for several hours at a temperature favorable for growth and toxin production of some pathogens. These statements should not be construed as indicating that disease-producing organisms commonly are associated with cheese and similar products. Actually, in this country, rigid supervision of production, together with pasteurization, and the subsequent destruction of chance pathogens by the lactic acid formed in cultured milk products all contribute to make these foods some of the safest that are consumed.

Inhibition of lactic acid bacteria occurs in products other than Cheddar cheese. Types such as Limburger, brick, Roquefort or blue, and many others, may be affected in the same manner. Serious losses caused by phage have been suffered in the manufacture of cottage cheese. The same is true of cultured buttermilk. Phage has caused serious difficulty in the manufacture of some byproducts such as baby foods that employ a lactic fermentation. Unquestionably the losses due to this agent have been greater in the dairy industry than is generally realized. Many technicians and operators do not recognize phage outbreaks when they occur. Isolations of phage continue to be made from dairy plants that have never suspected it as a cause of their difficulties with slow acid production.

Phage attacks have been reported thus far for only 3 species of bacteria important in cheese and other cultured milk products. Two of these, *S. lactis* and *S. cremoris*, are widely employed as

single strain or mixed cultures for acid production in the above mentioned dairy products. A recent report (19) of isolation of a phage capable of lysing *Leuconostoc citrovorum* may explain sudden loss in aroma production noted in mixed cultures of lactic acid and aroma bacteria. *L. citrovorum* and *Leuconostoc dextranicum* are grown as a mixed culture in association with *S. lactis* or *S. cremoris*. The *Leuconostoc* species ferment citric acid in milk and milk products to acetylmethylcarbinol and biacetyl. The latter compound contributes the characteristic pleasing butter aroma or bouquet to many cultured milk products. Without the minute traces of biacetyl, they would taste harsh and flat.

A curious fact is the absence in the literature of any report of phage associated with *Streptococcus thermophilus* and lactobacilli like *L. bulgaricus* and *L. lactis* in the manufacture of Swiss-type cheeses and some fermented milks. These organisms have higher optimum and maximum growth temperatures than the streptococci commonly attacked by phage; in most other respects *S. thermophilus* closely resembles *S. lactis* and *S. cremoris*.

## PROPERTIES OF *S. CREMORIS* AND *S. LACTIS* PHAGE

### MORPHOLOGY

Electron microscope studies of phage strains active against *S. lactis* 122-4 indicate the particles to be sperm-shaped (3, 27). The average dimensions observed were as follows: diameter of head, about 70 mμ; length of tail, 150 to 160 mμ; width of tail, 20 mμ; and over-all length, 220 to 230 mμ. Nine strains of phage were so nearly alike that no morphological difference among them was discernible. Electron micrographs of phages associated with cells indicated the phages to be oriented with tail toward the bacterial cell.

### ESTIMATION OF PHAGE POPULATION

The common means of establishing presence and concentration of phage for lactic acid streptococci is by dilution-sensitivity tests and by plaque formation. In the sensitivity test respective dilutions of phage are inoculated to suitable indicator media such as broth, litmus milk, resazurin milk or methylene blue milk. The highest dilution inhibiting the culture in the milk or causing visible cell lysis (demonstrated by clearing of the medium) in broth provides an estimate of the phage population. In the plaque method sensitive cells plus phage are spread on the surface of agar plates. Small clear areas on the plates surrounded by normal growth following incubation indicate lysis of cells by phage in those areas, and this provides an estimate of the number of phage particles present.

### NUTRIENT REQUIREMENTS

Little is known of the nutrient requirements of various phage strains specific for lactic acid streptococci. The requirements may be different from those of the host cell. One study (5) indicates that certain phage strains for *S. lactis* will not reproduce in synthetic media deficient in calcium, although the host developed satisfactorily under the same conditions. The *S. lactis* host-phage system has been shown to require factors not entirely supplied by yeast extract (4). Potassium phosphate, potassium chloride, sodium chloride, calcium chloride, magnesium sulfate, and sodium acetate promoted lysis of host cells according to their efficiency in promoting phage adsorption by the host cells. Sodium citrate allowed maximum adsorption but inhibited lysis.

### EFFECT OF PH OF MEDIUM

Phage adsorption on *S. lactis* has been shown to be highest at pH 7.0 (3). Lysis was most rapid at pH 7.0, somewhat slower at pH 6.0 and 8.0, and almost completely inhibited below pH 5.0.

### PHAGE REPRODUCTION AT DIFFERENT TEMPERATURES

Hunter (10) found that phages for *S. cremoris* showed a wider diversity of reaction to temperature than their homologous organisms. The optimum growth temperature for *S. cremoris* usually is near 30° C. They generally are inhibited to some degree at 37° C. Some phage strains in this study developed as well at 22° as at 30° C. Most phage strains developed more readily at 30° C. than at 22° C. Some developed more readily at 37° C. than at lower temperatures and others were completely inhibited at 37° C. These results are significant from the standpoint of laboratory studies on phage reproduction. They may also explain in part why some phage strains cause more difficulty in Cheddar cheese where manufacturing temperatures range from 30° to 40° C. than in other products that employ lower temperatures for the growth of lactic acid bacteria.

### BURST SIZE

The burst size (average number of phage particles released per infected host cell) of *S. lactis* 122-4 was calculated by Cherry and Watson (4) to be about 70 plaque forming particles at 30° C. in tryptone yeast extract broth at pH 7.0. This

is lower than the yields reported for some other organisms, and might be increased under more favorable conditions.

### Heat Resistance

Results of a number of investigators indicate that phage strains for *S. lactis* and *S. cremoris* appear more resistant to heat than do their host cells (25). Nichols and Wolf (25) found that active phages usually did not survive 75° C. for 7.5 minutes, but many were not destroyed at 65° to 67° C. for 50 to 60 minutes. Most survived 70° C. for 10 to 15 minutes. Cells of *S. lactis* and *S. cremoris* do not survive 71.1° C. for 15 seconds. These results emphasize the necessity of high pasteurizing temperatures in preparation of milk for the bulk culture. A minimum exposure of 82° to 88° C. for at least 30 minutes is recommended for this operation with the above data in mind. Another significant fact emphasized by these results is that normal pasteurization temperatures employed for cheese milk will not destroy most phage strains entering the plant with farm milk.

### Destruction by Chemical Germicides and Ultraviolet Radiation

Wolf *et al.* (39) studied the effect on air-borne *S. cremoris* phage of hypochlorite, resorcinol and propylene glycol mists. They concluded that hypochlorites offered the most practical means of destroying air-borne phage. Recommended exposures indicated by the study were a fine mist produced by spraying 4 ml. of a 9 to 12 per cent available chlorine solution per 1,000 cu. ft. air space, at a relative humidity of no less than 70 per cent.

Prouty (28) reported destruction of phage for *S. cremoris* following exposure in solution to 200 ppm of quaternary ammonium compounds for a period of 2 minutes. Studies (26) on comparative effect of quaternary and hypochlorite germicides indicate the hypochlorites to be more effective in destruction of *S. cremoris* phages than quaternaries over a wide pH range. The results indicate that hypochlorites should prove superior to quaternaries for destruction of phage on plant equipment, utensils, and building surfaces. In a study of a number of germicidal substances, Hunter and Whitehead (14) found chlorine and permanganate the most effective against phage.

Experiments on effect of ultraviolet radiation of lethal wave length on *S. cremoris* and *S. lactis* phages (8, 30) indicate that destruction by ultraviolet may be accomplished. However, the long time exposures required at relatively short distances from the ultraviolet lamp suggest this agent to be impractical for destruction of phage in the dairy plant.

### Phage Adaptation

The report of Nichols and Hoyle (22) indicates that a phage strain can be adapted to attack a previously resistant host. They were able to adapt a large number of phage strains to lyse selected strains of previously resistant *S. cremoris*. On the other hand many phage-organism combinations did not respond to the adaptation technique of exposing a resistant host to high concentrations of phage and isolation of the adapted phage from resulting plaques.

### Nascent Phage

The nascent phage phenomenon apparently occurs with lactic acid cultures (1, 10, 24). A nascent phage is one that normally will not attack an organism. However, if a strain of organism sensitive to that phage is also present, the phage may lyse both strains. The nascent phage reaction apparently is not common, but does present a potential hazard when two or more strains of lactic culture are mixed in bulk culture or in the final cultured milk product.

### Lysogenesis in Lactic Acid Bacteria

Lysogenesis (the production and liberation of phage by a host cell without lysis of that host cell) has never been conclusively demonstrated in cultures of *S. lactis* or *S. cremoris* (22).

### Phage-Carrying Strains of Lactic Acid Streptococci

Hunter and Whitehead (14, 15) have shown that cultures of lactic streptococci partially resistant to a specific phage strain may grow in association with that strain. As a result of an apparent blocking effect of that phage, the organism is protected from attack by other races of phage. Such cultures have been employed in commercial plants, but after several months other phages appear that attack the phage-carrying cultures. Hunter (12) has also reported that plating out cultures of lactic streptococci on agar and picking resulting colonies will free the organisms of all phage particles.

### Phage Typing

Nichols and Hoyle (22) have reviewed the studies on attempts to establish phage types of lactic acid streptococci. In a comprehensive series of experiments they succeeded in establishing 11 phage types or patterns for *S. lactis* and *S. cremoris*. In extending these studies they were

able to divide the majority of the phages into three serological groups by means of antiphage sera. The knowledge of phage types was believed of value in determining which commercial starters should be employed in cheese plants troubled with certain strains of phage.

### DEVELOPMENT OF PHAGE RESISTANT SECONDARY STRAINS

A number of workers have reported development of resistant secondary strains following phage lysis of a sensitive lactic acid culture (1, 12, 20, 31, 34, 35, 38). The use of such strains for starter cultures has been less successful than might be expected. In some studies these strains have proved less active in acid production than the original sensitive host strain. The apparent great number of phage strains existent also has resulted in an eventual attack of the resistant secondary strains. In some specific instances the development of resistant secondary strains has been successful in coping with polyvalent phage strains established in certain dairy plants.

### SOURCE OF PHAGES FOR THE LACTIC ACID STREPTOCOCCI

Nichols and Hoyle (22) have reviewed investigations on this subject. Phages for the lactics have been isolated from raw milk entering the plant, from various locations and pieces of equipment in the plant, from cheese, and from by-products such as whey powder. One worker, Maze (18) insists that phages for lactic acid streptococci are formed in the intestinal tracts of hogs. His claims have not been entirely substantiated by other workers.

### DETECTION OF PHAGE

Methods of detection and isolation are summarized in a number of reports (1, 2, 6, 7, 11, 12, 20, 22, 24, 25, 34, 36). The presence of bacteriophage in a starter culture or cultured-milk product may be suspected whenever the lactic acid bacteria suddenly slow up or completely fail to grow. Usually in such cases, a starter culture from a different source, containing other strains of lactic acid bacteria, will provide temporary relief from the difficulty—provided that no phage-strain specific for the new culture is present. In some cases, several strains of phage may be present in the plant. The phage sometimes may be one of multiple specificity and thus may be able to attack a number of different strains of starter organisms.

A few simple tests may be employed in the plant to provide presumptive evidence of the possibility of phage. A few drops of fresh starter may be added to about 10 ml. of sterile skim milk at 21° C. and if the milk fails to coagulate in 24 hours, the possibility of phage in the starter exists. If a second tube of milk is inoculated in the same manner and incubated at 30° to 37° C., microscopic examinations of the contents can be made at intervals over a period of about 8 hours. If the organisms begin to multiply and then lysis (disintegration of cells) is noted, the evidence is strong that phage has attacked and destroyed the bacteria. If phage is suspected in cheese manufacture, duplicate tubes or small bottles or flasks containing sterile skim milk may be inoculated with about 0.5 per cent of fresh starter. One of the duplicate containers then may be inoculated with 2 or 3 drops of whey from a suspicious vat. The other container serves as a control. The cultures may then be incubated at 86° F. for 6 hours and titratable acidity determined. If the acid developed is significantly greater in the control container, the presence of phage in the whey is strongly suggested.

The most certain method of demonstrating the presence of phage in a culture or product is to pass it through a bacteriological filter that will remove all microorganisms. At the same time, the culture suspected of attack must be plated on agar and the growth from a number of individual colonies transferred to sterile milk or broth to obtain single strains of the culture organisms. Duplicate sterile tubes of milk or broth then may be inoculated with the single strain cultures and a drop or two of filtrate added to one tube. If the control tube develops acid in significantly greater amounts or at a faster rate than the tube containing filtrate, there is a strong possibility of phage. The titer, or concentration of phage in the filtrate, may be determined by noting the presence or absence of inhibition in tubes of milk or broth inoculated with the single strain and varying dilutions of phage. It also is possible to demonstrate phage by smearing or inoculating a single strain culture and filtrate on agar plates and observing for plaque formation.

The growth from the above broth or milk may again be passed through a sterile filter. If the inhibitory substance can be increased or maintained in concentration by successive filtrations and periods of growth on a susceptible culture, it is bacteriophage. If the inhibitory substance is diluted out and gradually becomes weaker by such successive passages through the filter, it may be an antibiotic. Heating an inhibitory filtrate to 100° C. for 5 minutes also may aid in establishing whether it contains phage or antibiotic since phage is inactivated by this exposure and antibiotics usually are not.

The phage may be isolated from plaques with a sterile needle. Usually three successive passages through plaques with a transfer each time to fresh susceptible single strain culture will purify a phage strain. The resulting strain of phage may then be a single strain or race, or may be of multiple specificity.

### Practical Control Methods

The control of bacteriophage in many small, scattered plants throughout the country represents a difficult problem. Recommendations for the prevention of phage outbreaks have been presented by many workers (1, 6, 7, 15, 21, 22, 23, 36, 37, 39).

Since bacteriophage develops upon susceptible bacteria, it will be present not only in cultures or cultured milk products but also on growing organisms on equipment. It may lodge on floors, walls, ceilings, dust, and may even be carried on the hands and clothes of workers. Apparently droplet infection from the contaminated product, especially whey, tends to spread it around the plant and even into the starter laboratory, if it is located near the processing room of the plant. Whey separators are an especially difficult problem because they throw a fine atomized mist over the plant.

One measure found helpful in some plants has been a thorough cleaning followed by hypochlorite treatment of floors and all equipment that comes in contact with the product. Brushing, soaking, or thoroughly spraying all equipment and tools before use with 500 ppm hypochlorite solution is recommended where phage outbreaks occur. Another measure employed to reduce droplet infection is to spray the entire processing room with hypochlorite at the rate of at least 4 ml. of a 9 to 12 per cent solution per 1,000 cubic feet. The relative humidity of the room should be at least 80 per cent, if possible, for most effective penetration of the chlorine.

Some plants have reduced phage outbreaks and improved uniformity of starter cultures in general by obtaining mother culture daily from a central laboratory. This system greatly reduces the danger of phage contamination of the mother culture and enables one laboratory to maintain closer control over the quality and selection of starter strains than would be possible in scattered small plants. In some instances the mother culture is sent by air express from the central laboratory.

Another measure consists of removing the starter laboratory to some part of the plant away from the processing room, to reduce the chances of contamination of the culture. In some cases the starter laboratory has been set up some distance away from the plant. Elaborate precautions, such as means for sterilization of the room, maintaining positive air pressure in the room to prevent air currents carrying phage in, and the use of specially-constructed culture vessels with a small opening for inoculation and water seal of the lid also have been employed.

If several different cultures can be carried in the laboratory, they can be rotated in such a way that one or two are used one day, another combination the next, and so on. In Cheddar cheese manufacture, as many as 8 or 10 strains may be carried. Two cultures are grown separately and mixed at the vat on the first day, two other strains the next, and so on. Then the original two are used again and the rotation is repeated. This tends to prevent a build-up of phage for one culture day after day in the plant.

Where facilities are available, tests may be run on whey or other products to determine whether or not phage is accumulating for a certain culture. As soon as evidence indicates such an accumulation, another culture is introduced.

Strains of lactic acid bacteria may be made resistant to bacteriophage by repeatedly exposing them to phage and growing the survivors. Such strains may be resistant to numerous phage types, but the possibility of attack by another phage specific for them always exists.

The recent observations of Hunter and Whitehead (14, 15) relative to protection of a starter from other phage strains by growing it in association with a selected phage strain has suggested a possible protective mechanism for the culture; however, as pointed out by the authors, two possibilities may arise in such a circumstance: the culture may lose the protective phage and thus have no means of blocking out other phages capable of attacking it, and observations indicate that occasional phage strains may be encountered that will attack the phage-carrying culture.

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# Insect Transmission and Control of Plant Viruses

J. H. Freitag

INSECTS are the most important means for the field spread of plant viruses, the great majority of which are naturally transmitted only in this way. Plant viruses are also transmitted in a number of ways other than by insects, but these methods of spread are important only for a few exceptional diseases. The tobacco mosaic virus and potato X virus are transmitted by mere contact of diseased and healthy plants. The tobacco mosaic virus is also transmitted by handling and cultivation, especially in tomatoes. Viruses like big vein of lettuce and wheat mosaic are transmitted through the soil, and insect vectors may not exist for these two diseases. A few viruses like bean mosaic, lettuce mosaic, and squash mosaic are transmitted through seed as well as by insect vectors. However, transmission of viruses through seeds is not common. Vegetative propagation also provides a means for spreading viruses, but this method is important primarily only in a few crops such as potatoes, fruit trees and strawberries.

Experimentally all plant viruses are transmissible by grafting, and probably a majority of them by mechanical inoculation of plant sap from infected plants. A number of plant viruses also have been transmitted from diseased to healthy plants by means of the plant parasitic dodders.

The statement that plant viruses are transmitted by insects should not be interpreted to mean that any or all insects will transmit a particular virus, but rather that a certain insect species, or group of closely related insects, will transmit a specific virus. When one wishes to explain the field spread of plant viruses it becomes necessary to consider such things as species of insect involved, life histories, habits, host plants, movements, causes of population fluctuations and other ecological factors.

The aphids or plant lice have been demonstrated to transmit more viruses than any other group of insects. The green peach aphid, *Myzus persicae* (Sulz.), has been reported to transmit more than 50 different viruses. The potato aphid, *Macrosiphum solanifolii* (Ashmead), is next in importance and has been reported to transmit 30 viruses while the melon aphid, *Aphis gossypii* Glover, and the lily aphid, *Myzus circumflexus* (Buckt.), transmit at least 16 viruses.

The viruses transmitted by insects may be broadly classified into two groups, the nonpersistent and the persistent viruses (Watson and Roberts 1939). The nonpersistent viruses comprise the largest known group and are characterized by the fact that the vectors retain the ability to transmit this type of virus for a very short period, usually only a matter of a few hours. These viruses are usually mechanically transmissible and there is little specificity of their vectors. Some nonpersistent viruses may be acquired by their aphid vectors in only a few seconds feeding and may be immediately transmitted to healthy plants. However, the virus is rapidly lost by the insect when feeding on healthy plants.

Watson (1938) demonstrated that starvation before feeding on a diseased plant greatly affects the efficiency of aphids to transmit nonpersistent viruses. The aphid vectors of these viruses become most efficient when, following a period of fasting, they are fed for only a minute or two on infected plants and transferred directly to a healthy plant. As the feeding period on diseased plants is increased there is a very rapid and marked decline in the efficiency of the insect. The vectors usually do not retain infectivity for more than a few minutes when fed continuously on healthy plants, but if they are fasted following the acquisition feeding, they will retain the virus for a longer time.

The efficiency of single green peach aphids transmitting sugar beet mosaic virus can be increased by a period of starvation preceding the acquisition feeding, from 10 per cent for the unstarved aphids to 70 per cent or more for aphids starved for 15 minutes or more (Sylvester 1949). The green peach aphid has been demonstrated to be capable of acquiring the sugar beet mosaic virus in as short a period as 10 seconds and can infect a healthy sugar beet in as short a time as 10 seconds.

The second group of insect transmitted viruses, the persistent viruses, include some which are aphid transmissible, but in these cases they are acquired only after longer feeding periods (Watson 1940). The longer the time the aphids spend on a virus source and on the healthy test plant, the more likely it will be that transmission will result. Persistent viruses are generally not transmissible by mechanical inoculation of sap. The aphids are not usually immediately capable of transmitting the virus following a short feeding on a virus source plant. A period of time

must first elapse which has been called the latent period. Once the aphid becomes infective following the latent period, it usually retains the ability to transmit the virus for the remainder of its life. The persistent viruses have greater specificity among their aphid vectors and each is usually transmitted by one or only a few species of aphids. The same aphid species may transmit both persistent and nonpersistent viruses indicating that the differences in their vector relationships may arise solely from differences in the properties of the viruses.

As a group, leafhoppers are second only to aphids in their ability to transmit viruses. Oman (1949) lists 69 species of leafhoppers as being vectors of plant viruses. Although certain aphid species may transmit more than one virus, very rarely is a species of leafhopper reported to transmit more than one virus. Leafhoppers are more specific in their transmission than aphids, although recent work has demonstrated additional vectors for a number of viruses such as the work on aster yellows by Severin (1948) which indicates as many as thirty species of leafhoppers may be vectors of this virus. There are still a number of viruses, such as sugar beet curly top virus, for which only one or a few vectors are yet known.

The leafhopper transmitted viruses, all persistent viruses, are again characterized by a more or less definite latent period and a long retention of the virus by the vector. Viruses of this group are not generally mechanically transmitted. These viruses can be acquired by the vector in as short a feeding as one minute and inoculated into a healthy plant in a similar period, but between these two periods there is a delay in the development of the infective capacity of the insect. This delay is known as the latent period. The latent period may be as short as 6 hours in the leafhopper vector of streak of maize virus, *Cicadulina mbila* Naude (Storey 1928) to 14 days for the corn stunt virus in *Baldulus maidis* (DeL. and W.) (Kunkel 1948). The curly top virus has been demonstrated to be retained in the beet leafhopper, *Circulifer tenellus* (Baker) for 167 days without the leafhopper's having access to a virus source (Freitag 1936).

The long latent period, often referred to as an incubation period, and the long retention of the virus in the leafhopper have been considered to be evidence that the virus multiplies within the bodies of the vectors. Various theories have been proposed to explain the latent period. The first suggests that the latent period represents the time it takes for the virus to multiply to sufficient concentration to permit the insect to infect a plant. Another suggestion is that the latent period represents the time necessary for the virus to pass through the gut wall into the blood and thence to the salivary glands where it can be ejected. The long retention periods have been interpreted as being possible only because the virus is continually multiplying within the body of the insect.

The theory that plant viruses multiply in the bodies of leafhoppers has received considerable support from the work of Fukushi and Black. They have demonstrated that viruses may be transmitted from leafhoppers to their offspring through the egg. Fukushi (1940) has shown that the rice dwarf virus can be transmitted through the egg of the leafhopper vector for seven generations although the insects have no access to a virus source. Black (1949) demonstrated that the clover club leaf virus can be transmitted through 15 generations over a period of 4 years without loss of infectivity by the leafhoppers. If the virus were considered not to have multiplied, the estimated minimum dilution of the virus in the insect has been calculated to exceed $10^{-17}$. This dilution is far beyond the limit of dilution that any virus could stand and the best explanation for these results seems to be, at least in this instance, that the virus had multiplied in the insect.

The work which has been done on the curly top virus and the beet leafhopper suggests that the virus does not multiply in the vector, and that they contain no more virus than they take up while feeding on an infected plant (Freitag 1936, Bennett and Wallace 1938). This virus is gradually and irregularly given off in the salivary secretions. It has been shown that the length of time the leafhoppers remain infective and the number of infections they are capable of producing depend upon the length of time they were fed upon the source of virus. They may become infective after a few minutes' feeding, but if they do so they do not remain infective for long and produce only a few infections. If they are fed for long periods (hours or days) on inoculum they produce many infections and they may retain the virus during their entire lives. These results do not support the multiplication hypothesis.

Thrips are known definitely to transmit only one virus, that of tomato spotted wilt (Sakimura 1947). Three species of thrips have been demonstrated to be capable of transmitting this virus.

The disease has a world-wide distribution and was first investigated in Australia, later in England, Hawaii, South Africa, South America, and in the continental United States. Insect-virus relationships investigated indicate a 5 to 7 day latent period in the thrips, and a long retention of the virus by the vector for 24 to 30 days and sometimes for the life of the insect. The most unusual fact involved in the transmission of the tomato spotted wilt virus is the failure of adult thrips to acquire the virus. Only the larval thrips are capable of acquiring the virus. Upon the completion of the latent period, the larval thrips transmit the virus and continue to remain infective for long periods even after they emerge as adults. Thus, adult thrips can be vectors only when they acquire virus during the larval stage.

Five species of mealybugs have been shown by Posnette and Strickland (1948) to be the vectors of swollen shoot virus, the cause of a serious disease of cacao in the Gold Coast of Africa. They acquire the virus in less than 4 hours' feeding on an infected plant and can transmit the virus to healthy plants in less than 3 hours' feeding. The virus does not persist in the mealybugs. They are found in close association with ants, and this association is a very significant one in relation to the spread of the disease. The ant-attended mealybugs occur throughout the canopy of the trees and are covered by connecting earth shelters built over them by the ants. The ants carry the mealybugs about in the earth shelters and from one tree to another. The virus spreads most rapidly from tree to tree when the leaf canopies of the trees are in contact.

Whiteflies are known to transmit several virus diseases of plants. Costa and Bennett (1950) have shown that the mosaic disease of *Euphorbia prunifolia* Jacq., is caused by a typically persistent virus. The whitefly, *Bemisia tabaci* Genn., can acquire the virus only after a 30-minute feeding period on a diseased plant and can infect healthy plants in feeding periods of 10 minutes or longer. The latent period was 4 to 5 hours and the virus persisted in the whitefly vector for at least 20 days.

Although a majority of plant viruses are transmitted by insects with sucking mouthparts, recently some reports have appeared which indicate there may be a small group of viruses which are transmitted by insects with chewing mouthparts. The interesting fact is that some of these viruses are apparently not transmitted by insects with sucking mouthparts even though they are readily transmissible by sap inoculation.

Dale (1949) demonstrated bean leaf beetles to be efficient vectors of the cowpea mosaic virus while aphids have failed to transmit this virus. Single beetles acquired the virus after only 3 minutes' feeding on a diseased plant and when transferred directly to healthy plants for a similar period of feeding, succeeded in infecting 30 per cent of the plants on which they fed. The virus was retained by the beetles for 6 days following a feeding period of only a few hours on a diseased plant.

The turnip yellow mosaic virus has a rather unique group of vectors in that it has been shown by Markham and Smith (1949) to be transmitted by 5 species of beetles, 2 species of grasshoppers and by the common earwig. The virus is not transmitted by aphids, plant bugs or by caterpillars. Larvae of the mustard beetle acquired the virus in feeding periods of 1 to 10 minutes on infected plants and retained the virus for a period of 72 hours. Larvae bred on infected turnip yellow mosaic plants, however, infected a higher percentage of plants than those fed only 48 hours on a source of virus. These results suggest a correlation between the length of feeding on infected plants and the duration of the infective power and the number of plants infected. The virus was not retained during the pupal stage of the beetle.

The obvious assumption for the explanation of the mode of transmission of the turnip yellow mosaic is that the virus is carried mechanically on the jaws of the beetles, but this is not borne out by the experimental results which indicate that often a 24-hour period must elapse after the insects have fed on a virus source before they are capable of infecting healthy plants. A more likely explanation for the mode of transmission seems to be that the flea beetles lack salivary glands and that they regurgitate part of the contents of the foregut to aid digestion. During the regurgitation process the virus is returned to the plant and infection takes place. The suggestion is made that the insects with salivary glands such as the aphids and caterpillars probably do not regurgitate and consequently do not transmit the virus.

Anatomical investigations of the foregut of the green peach aphid indicates the presence of a suboesophageal valve which would prevent the regurgitation of food material in the crop. In the adult flea beetles and the larval mustard beetles the valve is located farther along in the alimentary tract between the crop and the midgut and therefore does not prevent regurgitation.

Squash mosaic virus appears to be a transition virus between the typical nonpersistent aphid borne viruses such as cucumber mosaic and the viruses like turnip yellow mosaic and cowpea mosaic which are transmitted only by insects with chewing mouthparts. Squash mosaic virus is most efficiently transmitted by cucumber beetles, but it has also been transmitted by aphids (Freitag 1941). This virus is one of the few insect transmitted viruses to be purified and crystallized by alcohol precipitation and high speed ultracentrifugation (Takahashi 1948). Electron microscope study showed that the virus particles are spherical.

Transmission experiments indicate that the striped and spotted cucumber beetles are the most efficient vectors of the squash mosaic virus and that they retain the virus for a period as long as 20 days. Five of the 10 species of aphids tested have been demonstrated to be inefficient vectors of the squash mosaic virus, while the other five species failed to transmit the virus in the tests conducted.

Attempts to control virus disease spread by the application of insecticides have been somewhat successful in a number of instances, but in others they have failed to give desired results. The best solution to the problem of efficient and complete control of virus disease does not yet seem to be in the application of insecticides, because of the near perfect control of the insects required in order to stop the spread of viruses. The work of Watson (1937) in England on virus diseases of henbane grown for medicinal purposes had indicated that weekly application of a nicotine spray during June and July reduced the spread of the virus approximately 50 per cent. The yield of henbane was significantly increased, enough in fact, to justify the cost of the control measures. Similar results were obtained by Stubbs (1948) in Australia in an attempt to control the aphid spread of a persistent virus disease of carrots. Weekly applications of a DDT emulsion for eight weeks resulted in excellent control of the aphid vector, but reduced the percentage of diseased plants only from 100 per cent in the check to 68 per cent in the sprayed plots. The yield of carrots was increased sixfold. This made the undertaking profitable.

Insecticides applied to control aster yellows virus on lettuce and carrots have been somewhat successful. The application of pyrethrum and derris dusts to lettuce in New Jersey by Pepper and Haenseler (1939) resulted in a 98 per cent reduction in the leafhopper population and an 89 per cent reduction in the amount of aster yellows disease on the lettuce crop. The spraying and dusting of the carrot crop with DDT in New York by Hervey and Schroeder (1949) has resulted in a 90 per cent reduction in the leafhopper population and an 80 per cent control of carrot yellows. Repeated applications were necessary to obtain these results. In many cases the leafhopper populations increased rapidly following treatment and approached the original populations in a week or 10 days. This indicated a continuous movement of vectors into the carrot fields.

The application of DDT dusts and sprays to potato plants in Maine by Bronson *et al.* (1946) has resulted in excellent control of the aphid vectors, but has not given the desired control of the potato viruses. Similar results have been obtained in California in attempts to control the spread of the tomato spotted wilt virus by applying DDT dusts against the thrips vectors. Although a good kill of the thrips was obtained, only slight disease control resulted. Gardner and Michelbacher (1945) applied DDT successfully to control thrips in the greenhouse and appreciably reduced the spread of the tomato spotted wilt virus.

The most likely explanation for the failures to control the field spread of plant viruses appears to be that the vectors are continually moving into cultivated fields from weeds and other outside sources of infection following the insecticide application. The interval of time elapsing between the applications of the insecticide apparently allows the invading vectors to cause infection before they are eliminated by the next application.

Insecticides applied to control the spread of cantaloupe mosaic in the Imperial Valley of California by Dickson *et al.* (1949) resulted in no measurable reduction of the disease. The green peach aphid had bred up in tremendous populations on the sugar beet crop and during the spring migrated across the valley in enormous numbers. This transient population was provided optimum conditions for the spread of nonpersistent viruses. The aphids were starved during flight and they made only short stops of approximately 40 seconds on the melon plants. The aphids started feeding almost immediately, and following the short feeding period moved on to other plants to repeat the process. During the peak of the aphid flights counts made indicated that an average of 50 aphids per minute landed on each melon plant and a similar number departed. The

aphids moved across the fields in a succession of short flights and apparently fed for short periods on a large number of plants. These conditions resulted in ideal conditions for efficient transmission of the virus by the aphids and made it especially difficult to control the spread. The fact that no appreciable number of aphids could be found following insecticide application in either the check or dusted plots indicated that the aphids had not established themselves on the melon plants. The only control practice likely to stop the spread of cantaloupe mosaic would be to reduce the aphid population on the sugar beets, which were the source of the aphid flights, or to use an effective repellent to prevent the aphids from feeding on the cantaloupes. Neither of these practices has been successfully carried out.

A rather interesting method of controlling a plant virus was started in California during the fall of 1931, when several beet sugar companies started spraying operations in the foothill breeding areas of the beet leafhopper in an attempt to reduce the damage to sugar beets caused by the curly top virus.

The beet leafhopper breeds continuously during the summer months and usually produces three broods. The third brood of leafhoppers matures in October in the cultivated areas of the San Joaquin Valley and these insects leave, many of them finding their way to the foothill areas of the Coast Range Mountains bordering the west side of the valley (Severin, 1933). Russian thistle is the most important summer and fall host plant of the beet leafhopper. Since there is a lack of rainfall during the summer months, the only plants available for the leafhoppers to feed on in the foothills are the perennials and the late summer annuals which occur mainly in wash bottoms. During this time the leafhoppers are often forced to congregate on the few host plants available and it is against these concentrations that insecticide sprays have been applied during the fall months in an attempt to reduce the overwintering population. Following the fall and winter rains the annual weeds germinate and the leafhoppers lose little time in dispersing to the sparse vegetation located on favorable southern slopes. During the winter the females deposit their eggs and one or two broods develop in the foothill areas before the leafhoppers move back to the cultivated areas in the valley during April and May.

The control program carried out by the sugar companies consisted of spraying the fall and winter concentrations of beet leafhopper populations in washes and slopes of the foothill areas (Cook, 1943). This would reduce the overwintering population before it dispersed on the winter annuals. During the spring a second spray program was carried out against high populations occurring on annual plants to cut down the population which would later move into the cultivated areas. In addition to these spray programs, control of Russian thistle was practiced and huge acreages of this favorite summer and fall host of the beet leafhopper were eliminated (Wallace, 1948). Reduction of Russian thistle is believed to be essential if any progress is to be made in keeping down the leafhopper populations.

It has become very difficult to evaluate the foothill control program directed against the beet leafhopper. The program was operated by the sugar companies from 1931 to 1943, when they discontinued the program because they had come to rely upon varieties of sugar beets resistant to the disease. The California State Department of Agriculture took over the work in 1943, because it was felt that the increased tomato, melon, flax and spinach crops needed continued protection against the ravages of the curly top virus disease. The work has been continued by the State Agriculture Department up until the present. Previous to this program of foothill control of the beet leafhopper and its weed host, Russian thistle, there had been periodical outbreaks of curly top which had been disastrous not only to the growers of sugar beets, but also to the tomato, melon, and spinach crops. Since the inauguration of this control program there have been no serious outbreaks of the virus, such as occurred in 1919 and 1925, although there have been years when certain crops such as tomatoes have suffered considerable damage as a result of curly top infection. The general impression is that the program has helped considerably in the reduction of curly top damage, but it is also possible that environmental conditions and cultural practices have not been favorable for the development of high leafhopper populations and the resulting severe curly top damage.

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# Some Significant Findings From Research on Poultry Viruses

J. R. BEACH

DISEASES caused by one or another of the viral pathogens of poultry which are discussed in this paper have, at various times during the past thirty years, become so prevalent and devastating as to threaten seriously the future of poultry raising on a commercial scale. In fact, it is doubtful that specialized poultry raising, with its resultant concentration of large numbers of birds in relatively small areas, could have survived if effective procedures for the control and prevention of these diseases had not been forthcoming. Intensive and sustained study of poultry disease had its inception about 1915 in certain agricultural experiment stations. With the recog-

nition that poultry raising had become an important part of the livestock industry came a rapid expansion of poultry disease research in the experiment stations and certain veterinary colleges. It is by the group of so-called "poultry pathologists" thus created that the major portion of research on virus diseases of poultry has been done. As knowledge of these viruses grew, men working with human viruses at universities and medical research institutes came to realize that poultry viruses might serve as virus types for studies of a fundamental nature, and that their natural host was an excellent laboratory animal. Thus the findings from research on poultry viruses are of both economic and scientific significance. They have provided means for controlling the diseases of poultry produced by the viruses and have contributed to the knowledge of virus behavior. The viruses to which reference has been made, and which will now be discussed in more detail, are those of fowl pox, infectious laryngotracheitis, infectious bronchitis, and avian pneumoencephalitis, more commonly termed Newcastle disease. The first named was already well-known and prevalent prior to 1915; the other three have appeared and been identified subsequently.

## FOWL POX

This is an ubiquitous disease of poultry of nearly world-wide distribution. The viral cause was first demonstrated by Marx and Sticker[1]* in 1902. It was firmly established, causing severe losses on poultry farms in the United States in the early years of this century. Susceptible species include chickens, turkeys, pigeons, canaries, and several wild bird species. The occurrence of it in waterfowl has also been reported. A number of workers have attempted to divide pox virus into different strains on the basis of bird hosts. Thus reference has been made to fowl (chicken) pox, turkey pox, pigeon pox, and canary pox strains of the virus. The various reports of such studies indicate that mono-, bi-, or tri-pathogenic strains of the virus may exist. The diversity of the findings in this report, however, suggests that seeming host-specificity may be related to a strain of the virus having become so firmly adapted to a certain bird species that it appears to be pathogenic for that species only, but that this specificity can be broken down by continued passage on to another species. Both success and failure have resulted from attempts to establish the validity of this concept. Some failures may have resulted from lack of persistency. For example, Beach and Lubbehusen[2] in attempting to enhance the pathogenicity and antigenicity for chickens of two pigeon strains of virus by passage in chickens, observed some, but not marked, increase in these respects at the 28th passage, but little further change was noted in additional passages through the 85th. During this series of chicken passages, the virulence of the virus for pigeons did not appreciably decline. From the 86th to the 105th chicken passage, however, the virulence of the virus for chickens markedly increased, and this was accompanied by a corresponding decline in its virulence for pigeons. To prove that the somewhat abrupt change was not the result of accidental contamination with a chicken strain of virus, the series of chicken passages were repeated, beginning with virus of the 84th passage, and the results were the same. The virulence for pigeons was largely restored by one or two passages in that species of bird. We failed, however, in attempts to adapt a chicken strain to pigeons, which is contrary to results reported by Irons[3] and others.

Experiments in prophylactic vaccination against the disease were undertaken in 1914. The vaccine first tried consisted of a saline suspension of finely ground comb lesions with a chemical added to inactivate the virus. This vaccine, although variable in effectiveness, exhibited enough protective effect that, within three years, it was being produced on a commercial scale and extensively used in certain areas. This is of particular significance because it marks the beginning of acceptance, as a routine practice by owners of poultry flocks regardless of the number of birds they contained, of a disease-control procedure requiring the treatment of each individual. It was not easy to convince poultrymen of the practicability of a procedure which required not only individual handling but also making a hypodermic injection, because heretofore they had held to the belief that the only proper way to give medication of any sort to chickens was with feed or in the drinking water. In 1923 it was found that application of virulent fowl pox virus to feather follicles of the leg from which the feathers had been plucked, or pricking the skin with a pointed instrument moistened with fowl pox virus produced only a localized lesion of short duration. The pioneer work in this vaccination procedure was done by Dr. W. T. Johnson and Dr. E. M. Dickenson at the institution where we are meeting today. This finding brought into being the fowl pox

* See References, page 43.

vaccination method now in general use, which has been the means of reducing to a negligible size the loss to the poultry industry from a once highly destructive disease.

Fowl pox virus is a prolific producer of cytoplasmic inclusion bodies in epithelial cells of the skin. For this reason it is commonly used to demonstrate cellular inclusions to students in pathogenic bacteriology and virology courses. Woodruff and Goodpasture[4 5] showed that the inclusions consisted essentially of an aggregation of elementary or virus bodies enclosed in a capsule of lipo-proteid composition. In accomplishing this the pox lesion tissue was subjected to tryptic digestion by which the inclusion bodies were completely separated from the tissue, thus enabling the isolation of individual inclusion bodies. These workers stated that a typical pox lesion was produced by inoculating the skin of a susceptible chicken with a single inclusion, and further that an inclusion body may contain as many as 20,000 elementary bodies, each of which is capable of inciting the disease in susceptible fowl. By electron microscopy, a number of research workers have found that morphologically the elementary fowl pox body closely resembles those of vaccinia and variola. Mathey[6] recently reported an interesting observation regarding inclusion body formation following inoculation of the combs of chickens and the chorio-allantoic membrane of chicken embryos with five commercially produced live-virus fowl pox vaccines and a laboratory strain of virus. Inclusion bodies were readily demonstrated in proliferating tissue cells of the comb and embryo membranes as early as the third day following inoculation with three of the vaccines and the laboratory strain. Mathey reports inability to demonstrate inclusion bodies, however, in lesions on either the comb or embryo membranes produced by two vaccines, but did find elementary bodies in the allantoic fluid of the embryos. The lesions which developed on the combs of the chickens regressed rapidly with little or no scab formation. The chickens, after the lesions subsided, were refractory to challenge with the laboratory strain of virus. Since the significance of this observation is not clear to the writer no interpretation is attempted.

Fowl pox virus has the distinction of being the one used by Woodruff and Goodpasture[7] in 1931 in their first demonstration of virus propagation in chicken embryos. The extent to which this method of cultivation has been applied to many other viruses and the boon it has been to virus and rickettsial research is too well known to require detailed comment here. It has provided the virologist with a tool comparable to the culture media of the bacteriologist. Without it, knowledge of viruses and virus diseases quite possibly would not have advanced to its present high level. It has afforded a simplified means of preparing several bacteriologically sterile live-virus vaccines. It has also proved useful as a culture medium for bacteria, fungi, and protozoa. The statement by Goodpasture[8] in 1940 that "the potentialities of the chick embryo method for studies of infection have only begun to be recognized" may still hold true today.

## INFECTIOUS LARYNGOTRACHEITIS

The report by May and Tittsler in 1922[9] concerning an outbreak of "tracheo-laryngitis" appears to be the first recognition of the disease which became termed laryngotracheitis. It was next recognized as a disease entity in California in 1924 by Beach[10] who termed it "infectious bronchitis." The finding, however, by Seifried[11] in 1931 that it attacked principally the larynx and trachea, and in that order, led to the adoption of the more appropriate name "laryngotracheitis." The original focus of the disease appears to have been the Central Western States. Operators and employees of poultry fattening stations in that area claimed it had been present there for many years, but losses caused by it were not serious previous to the fall of 1924. At any rate, the disease was with certainty carried to both the Atlantic and Pacific coasts by infected Midwestern market poultry and within a few years had become a national poultry disease problem. The first identification of the disease outside the United States and Canada was by Seddon and Hart[12], in Australia in 1935, and subsequently it has been observed in several European countries. The disease runs an acute course and usually terminates in death or complete recovery within a week after first symptoms are seen. The disease is fatal only when the accumulation of exudate and blood occludes the lumen of the larynx or trachea and suffocation ensues.

The cause of the disease was definitely established to be a virus by Beach[13 14] in 1930. The virus is present in abundance in laryngo-tracheal exudate of infected birds. It has been demonstrated in the blood, livers, and spleens of some birds, but its presence there is believed caused by the accidental entrance into the blood stream through the injured walls of the blood vessels of the larynx and trachea and does not imply any real involvement of the other organs. Virus host-

specificity is well exemplified by laryngotracheitis. Chickens and closely related pheasants are the only bird species that have been found susceptible to it. Several species of domesticated and wild birds have proved refractory to the virus and so too have rabbits, guinea pigs, and white rats. The virus was cultivated *in vitro* in Tyrode's solution —minced-embryo medium by Beach[15] in 1932, and in chicken embryos by Burnet[16] in 1934. All live-virus vaccine currently used for immunizing against the disease is prepared from infected chicken embryos. No immunologically different strains of the virus have been discovered.

In 1932 Hudson and Beaudette[17] made the unique discovery that the application of virus to the mucosa of the cloaca or bursa of Fabricius of susceptible chickens produces localized infection of short duration which does not spread to the respiratory organs or otherwise adversely affect the birds, and confers a solid lasting immunity against natural or artificial infection by the intratracheal route. Beach[18] reported inability to demonstrate virus in the bursa of Fabricius later than the seventh day following the intrabursal injection of virus. This time-interval corresponded to that during which an inflammatory reaction of the cloacal mucous membrane was visible. This was regarded as indicating chickens infected intrabursally did not become virus carriers. Komarov and Beaudette[19], on the other hand, reported having recovered the virus from the trachea of chickens as long as 16 months after recovery from the disease. They considered that recovered birds could serve as reservoirs of infection and cause a fresh outbreak in an oncoming susceptible population of young chickens.

Cloacal infection was quickly shown by Beaudette and Hudson[20] to provide a highly effective and practical means of vaccination for laryngotracheitis. The vaccination procedure consists in the application of virulent virus to the cloacal mucous membrane with a brush or by another, though little-used, method which consists of injecting virus into the bursa of Fabricius through a curved, blunt hypodermic needle. An unusual feature of this live-virus vaccination procedure is that highly virulent virus is preferable, because it produces a marked and easily recognizable inflammatory reaction or "vaccination take" of the cloacal mucous membrane; but otherwise has no more effect on the birds than a virus of low virulence. Commercial production of laryngotracheitis vaccine was soon started and it had immediate acceptance by poultrymen in areas in which the disease was well established. Laryngotracheitis by this time had become so prevalent, and the income from infected flocks so markedly reduced, that unless some means of preventing it had become available, it is doubtful that specialized poultry raising in some areas could have survived.

### INFECTIOUS BRONCHITIS

This disease was first studied and described by Schalk and Hawn[21] in 1931 under the title "An apparently new respiratory disease of baby chicks." By 1933 it had been recognized in California, Kansas, and Massachusetts and soon its distribution was found to be nation-wide. The probable reason for its not being identified earlier in some areas is that it was mistaken for laryngotracheitis which it closely resembles symptomatically. Originally the disease was thought to be confined to young chicks, but later it was found that chickens of any age are susceptible. Outbreaks in very young chicks are likely to be accompanied by high mortality. Chickens affected during the growing period, that is from 5 or 6 weeks to 5 or 6 months of age, ordinarily suffer little mortality, and their rate of development is not seriously retarded. Laying chickens, likewise, are not fatally affected but the disease has a profound adverse effect on both the number and quality of the eggs which are laid. This is considered by many to be the source of greatest economic loss from the disease.

In 1936 Beach and Schalm[22] demonstrated that the cause of the disease is a virus distinct from that of clinically similar laryngotracheitis. Beaudette and Hudson[23] found that the virus could be propagated in chicken embryos by inoculation onto the chorio-allantoic membrane. The virus was said to have no visible effect on the embryos at first, but after a few passages it developed the power to cause dwarfing and death of the embryos. With additional embryo passage the virus became more lethal to embryos and less pathogenic for chickens. Delaplane and Stuart[24] later found that if propagation in embryos was continued long enough the virus became so completely adapted to embryos as to be avirulent for chicks. Delaplane also reported that adaptation of the virus was hastened by making the inoculations into the allantoic cavity instead of onto the chorio-allantoic membrane. It is of interest that the modification of the virus was accompanied by a loss in antigenicity for chickens. Therefore, the modification did not make available a virus of low virulence suitable for preparing a live-virus vaccine, as might reasonably be expected. The embryo-adapted virus, however, has proved very

valuable for use in neutralization tests with serum of chickens which have recovered from a respiratory disease suspected to be infectious bronchitis. It is particularly valuable for differentiating infectious bronchitis from the clinically similar respiratory diseases, laryngotracheitis, pneumoencephalitis, and infectious coryza, and is routinely used for this purpose in many diagnostic laboratories.

### AVIAN PNEUMOENCEPHALITIS (NEWCASTLE DISEASE)

In 1940 numerous flocks of two to ten weeks old chicks in California were affected with a disease which began as a rapidly spreading respiratory trouble. In a few days after the onset some of the birds developed symptoms of involvement of the central nervous system, the ultimate number of such cases varying from a few to as many as half or more of the flock. This led to the erroneous conception that a nervous disorder of unknown origin was occurring coincidentally with infectious bronchitis. The respiratory and nervous symptoms, however, were soon determined to be manifestations of one disease. This was first known as a respiratory-nervous disorder but soon was given the name of "avian pneumoencephalitis" by Beach[25]. Despite the rapidly spreading nature of the disease in affected flocks, artificial transmission proved difficult and was not accomplished until late 1941. The cause of the disease was then shown by Beach[25] and Stover[26] to be a virus which could be readily propagated in chicken embryos. This virus was also identified,[25 27] with a previously unclassified, relatively nonfatal respiratory disease of laying pullets which had been prevalent in the state for at least seven years. The disease was quickly detected among the poultry in nearly all sections of the state. The first indication of existence of the disease outside California was the demonstration by Minard and Jungheer[28], in 1944, of significant concentrations of neutralizing antibodies for a California strain of virus in serum of the University of Connecticut poultry flock, in which no disease suggestive of pneumoencephalitis had been observed. The next known extra-California infection was in pullets at Western Washington Agricultural Experiment Station, the diagnosis being made by serological tests and challenge with virus of recovered specimens shipped to the University of California. During the winter of 1944-45 the disease was identified by Beaudette and Black[29] among both layers and chicks in New Jersey. As had happened earlier in California, because the low mortality and the respiratory symptoms were mistaken for infectious bronchitis, the disease was well established in the state before it was identified as pneumoencephalitis. Recognition of the disease in other sections quickly followed, and it has now been identified in all 48 states, the District of Columbia, and Territory of Hawaii. The means by which the disease became so widely distributed is, for the most part, quite obscure. There are some rather clear-cut instances of its having been carried from one section to another by baby chicks and young breeding birds. There has not, to the writer's knowledge, however, been any connecting link established between the occurrence of it on the west and east coasts except in one case of its possibly having been introduced into a flock in a Pacific Coast state by breeding cockerels from New England. How long the disease may have been present preceding its first recognition in any state is unknown because of the difficulty in differentiating it from other respiratory diseases and the unavailability in many states of adequate diagnostic facilities.

In culturing the virus in embryos it was found that, irrespective of the mildness of the disease in naturally infected chicks from which the virus was isolated, its virulence so increased that an acute fatal infection and types of lesions, particularly submucous hemorrhages in various portions of the digestive tract which are seldom if ever seen in birds with natural infection, were produced in chickens inoculated with minute quantities of infected embryo fluids or tissues. These findings suggested a possible relationship between the pneumoencephalitis virus and that of either Newcastle disease or fowl plague, two highly fatal virus diseases of poultry, neither of which was known to exist in the United States. To explore this possibility, antiserums for both diseases were obtained from England in 1943 and used in neutralization tests with cultured pneumoencephalitis virus. The infectiousness of the virus was not affected by the fowl plague antiserum, but neutralizing antibodies for the pneumoencephalitis virus were definitely demonstrated in the Newcastle disease immune serum.[30] A short while later these findings were confirmed by Brandly and coworkers.[31] The interpretation of this finding has been a somewhat controversial matter. The question involved was well expressed by Stafseth *et al.* in a committee report at the U. S. Livestock Sanitary Association meeting in 1944 which reads in part: "An important development in the field of poultry pathology . . . is

the report by Beach on the neutralization *in vitro* of avian pneumoencephalitis virus by Newcastle disease immune serum . . . whether this means that Newcastle disease and pneumoencephalitis are caused by the same virus or by two antigenically related viruses remains to be proved." There is, of course, "much that could be said on both sides," and only time will reveal which of the opposing concepts is correct. In the meantime the writer has preferred to use the name pneumoencephalitis, because this term clearly refers to the type of disease thus far experienced in the United States, rather than one with the devastating characteristics which Newcastle disease has exhibited in other parts of the world.

The mortality in outbreaks of pneumoencephalitis have been exceedingly variable and in general has been confined principally to birds which developed nervous symptoms. The death loss has tended to be greatest among chickens under a month old, although some outbreaks in mature flocks have resulted in the death of a large percentage of the birds. The average mortality has been so low, however, that many poultry raisers fear the disease more because of the loss resulting from decreased egg yield and deterioration in the market quality and hatchability of the eggs than that from death. Investigation of some recent outbreaks of a predominantly respiratory type of disease in flocks of 4 to 6 weeks old chicks, which were accompanied by high mortality from occlusion of the large bronchi with caseous exudate, revealed concurrent infection with pneumoencephalitis and infectious bronchitis. This is cited as an illustration of the difficult diagnostic problems presented by the group of virus respiratory diseases under discussion. Another factor which contributes to the difficulty in detection and diagnosis of the disease is that it may occur as an inapparent or subclinical infection. This has been detected in many flocks. For example, the findings from studies concerning a large breeding establishment indicated that inapparent infection had been present among the population for approximately two years before the first clinical evidence of it was observed.

As was first shown by Burnet[32] and Lusk,[33] Newcastle disease virus has the property of agglutinating red blood cells and antihemagglutinins are present in the serum of recovered birds. The same properties were demonstrated by Beach[34] and Brandly and coworkers[31] for pneumoencephalitis virus and antiserum and the HA and HI tests soon came into common use, respectively, for the identification of virus isolated in embryo culture and as a quick serological diagnostic test. Beach[34] in reporting on the experimental application of the procedures to pneumoencephalitis states that different strains of virus, i.e., virus isolated from different sources, showed variations in hemagglutinative ability that were unrelated to virulence of the virus. He also observed that, although serums with virus-neutralizing activity invariably were also hemagglutination inhibiting, those having like neutralizing antibody concentration varied widely in antihemagglutinin content. For example, a variation in HI titer of from 1:20 to 1:10240 was found in 36 serums all of which had a virus-neutralizing titer of $10^3$. The HI test has become a routine diagnostic procedure and a positive reaction is regarded as definite evidence of present or past infection in the donor of the serum. Brandly and coworkers[35] made the interesting finding that virus antibodies are present in the yolk of eggs laid by immune hens and also that chicks hatched from such eggs may have congenital passive immunity adequate to protect against the infection for the first 30 days, or a little more, of their lives. They stated further, that such chicks become susceptible to the disease at the termination of the passively immune period even though in the meantime they may have been exposed to the infection. The yolk of such eggs has proved to be a satisfactory substitute for serum in making HI tests.

The reports by Jungheer[36] and Van Roekel[37] of having isolated the virus from fresh eggs laid by infected hens suggested the possibility of egg transmission of the disease. DeLay[38] obtained more conclusive proof of this by demonstrating virus in the yolk sac of healthy-appearing 4-day-old chicks, from embryos dead on the 15th day of incubation, and from eggs which were found to be infertile on the 7th day of incubation. The chicks, and eggs, came from parent stock which was actively infected when the eggs were laid. Although these findings indicate that transmission of the disease through hatching eggs is possible, it is not believed to be a common source of infection in chicks. There is abundant evidence that eggs laid by recovered chickens do not carry the virus.

Transmission of the virus through the air has long been suspected because of the very rapid spread of the disease through a flock. DeLay, DeOme, and Bankowski[39] obtained definite proof that the infection could be transmitted by this route by demonstrating the virus in normal allantoic fluid through which a measured volume of

air of a poultry house containing an infected flock had been drawn; and also in dust collected from the air of the poultry house with a vacuum cleaner. In a more direct test of the infectivity of the air, the disease developed in normal chickens within 6 days after confinement in cages suspended from the ceiling of the poultry house where they were out of contact with any contaminated material that was not air-borne. These workers state that they were unable to find in the literature any previous report of virus having been isolated from air contaminated as a result of natural infection.

Attempts to develop vaccine for prevention and control of the disease were begun immediately following the first isolation of the virus in embryo culture. The vaccines first tried were prepared from fluids and tissues of infected embryos with formalin added to inactivate the virus. Extensive laboratory experiments and controlled field trials were conducted in California,[40] from 1942 through 1946. Similar studies were made elsewhere, particularly by Brandly *et al.*[41] These efforts failed to provide a vaccine which would give complete and lasting protection against natural exposure to the disease. An interesting observation was that the formalized virus was more active in preventing development of nervous symptoms than symptoms of respiratory involvement. Such vaccine has not been generally used by poultry raisers, although it was and still is produced by some commercial biological laboratories. Research workers were also endeavoring to find avirulent strains of the virus or methods of attenuating the virulence of it so that a live-virus vaccine which would solidly immunize could be provided. The accomplishments in this respect which have been reported are briefly as follows:

Komarov and Goldsmit[42] stated in 1946 that by 14 intracerebral passages in ducklings a virulent Palestine strain of Newcastle disease virus became so modified that chickens inoculated intradermally with it did not develop symptoms and were immunized.

Reagan *et al.*[43 44] in 1948 reported having adapted a California strain of virus to hamsters through serial passage by intracerebral inoculation. This brought about a progressive decline in the virulence of the virus for chickens.

The first report of field use of low-virulent virus was by Van Roekel *et al.*[45] who stated having obtained "strains of virus of low virulence which can be used by the stick method (piercing the web of the wing with a needle moistened with infected embryo suspension) to immunize sexually mature chickens without producing an active outbreak of the disease."

Clancy[46] and Markham[47] and associates, in 1949, reported the results of both laboratory and field vaccination trials with virus modified by propagation in duck embryos. They stated that the vaccine, administered by the stick method, was well tolerated by chickens above the age of 4 weeks, producing clinical infection in a negligibly small number. Although the infection readily spread to nonvaccinated groups, the resultant disease was said to be no more severe than that caused directly by the vaccine. This attenuated strain is one of those used in the commercial production of live-virus vaccine. Similar experiments and results were reported from Palestine by Komarov and Goldsmit[48] in 1947. Beach *et al.*[49], however, reported failure to obtain any alteration in the virulence of two California strains and one Montana strain of virus by 58 serial passages in duck embryos. (The passages were continued and the number has now reached 95.)

Beach *et al.*[49] at the same time reported having obtained progressive decline in the virulence of two highly virulent California strains through serial chicken-embryo passages by inoculation into the yolk sac instead of the allantoic cavity. This decline was apparent by the 21st passage and by the 73d passage the ability of the viruses to produce clinical infection appeared to be nearly lost; but this was not accompanied by a comparable loss in antigenicity. Attempts to restore virulence of the attenuated viruses by serial embryo passage by allantoic inoculation resulted, instead, in a still further decline in virulence. An interesting finding in both laboratory and field vaccination experiments is that the infection has invariably failed to spread from vaccinated birds to nonvaccinated susceptible contacts.

Bankowski[49 50] has succeeded in propagating the virus *in vitro* by using a modified Simms-Sanders[51] liquid medium. This cultivated virus has low virulence for chickens but not for embryos. The results of further work indicate that this may be a suitable method of propagating virus for use as a live-virus vaccine.

Beaudette[52], proceeding on the premise that naturally avirulent strains could be found, systematically made virulence tests with chickens of 105 strains which he had isolated in embryos. In this way he detected several which exhibited low pathogenicity for chickens and from these selected one which appeared particularly suitable for use as a live-virus vaccine. After thorough laboratory and field tests had demonstrated that mortality following vaccination of growing chickens with this strain was negligible, it was selected by a commercial laboratory for production of a live-virus vaccine which has been extensively used by poultry raisers.

The avirulent strain of virus most recently described is one encountered by Hitchner and Johnson.[53] The exact origin of this strain appears to be rather obscure. The pathogenicity of it is so low that it is tolerated by day-old chicks and causes no decrease in egg yield or other undesirable reaction when administered to laying chickens. In these respects it differs from the other low-virulent viruses described above, with the possible exception of the *in vitro* propagation virus of Bankowski. The durability of the immunity induced by this virus has not been determined. Nevertheless vaccine for administration by the intranasal route is being commercially produced. It is intended only for vaccination of very young chicks and susceptible laying hens for which protection is needed.

It is seen from the foregoing that the efforts of research workers to produce or discover pneumoencephalitis of sufficiently low virulence for use as a live-virus vaccine have been fruitful. A fortunate accompanying circumstance is that all of these strains have remained highly virulent for embryos. In general, vaccination for pneumoencephalitis with live virus has proved a satisfactory and effective immunization procedure.

Five cases of human infection with Newcastle disease or pneumoencephalitis virus, verified by virus isolation, have been observed[54 55 56]. The infection in each case consisted of a mild conjunctivitis of short duration. Three of these were among laboratory personnel working with cultured Newcastle disease virus in Australia, and two in the United States resulted from contact with chickens having natural pneumoencephalitis infection. Howitt *et al.*[57] reported having demonstrated neutralizing antibodies for pneumoencephalitis virus in the serum of children affected with a mild meningo-encephalitis and in adults showing respiratory symptoms. In a later report[58], however, Howitt stated that further studies had shown that the positive results of the neutralization tests with the virus were due to a nonspecific heat-labile factor in the human serums. Recently, Jungheer *et al.*[59] reported the presence of both neutralizing antibodies and antihemagglutinins for a California strain of pneumoencephalitis virus in the serum of a number of patients convalescing from mumps. Beyond stating that the finding "suggests a possible seriological relationship between the two viral agents" no interpretation of this finding was offered. The susceptibility of the conjunctival membrane of man to the virus seems to be clearly established. In view of the facts, however, that only two such cases related to contact with naturally infected chickens have been detected and that virus has been shown to be present in the air of poultry houses containing infected chickens, it would appear that conjunctival infection is likely to be produced only by bringing virus into intimate direct contact with the membrane as might happen, for example, if a person making an autopsy of a dead chicken should wipe his eyes with contaminated fingers.

The pneumoencephalitis virus has proved useful in research on the nature and behavior of viruses and in courses in bacteriology given to medical students. Dr. Ernest Jawetz, Associate Professor of Bacteriology, University of California, School of Medicine, summarizes its advantages in the following manner: "The teaching of virus infections to students suffers from difficulty to perform simple, clear-cut tests, and see the end results in a short time. This virus fills the gap because it 'does everything.' Size and shape of the virus can be demonstrated because it sediments readily by high speed centrifugation. It has high pathogenicity for the natural hosts, chickens and embryos, and regularly produces a typical pathological picture. Any tissue and fluid from infected embryos is highly infectious. The virus has high stability; will remain viable in the refrigerator for 1 to 2 weeks and at —70° C. indefinitely. It can be used in serum-neutralization tests, hemagglutination and hemagglutination-inhibition tests, and for removal of receptor substances from red blood cells. An additional factor also worthy of mention is that this virus is not a menace of consequence to laboratory personnel or students."

The three respiratory viruses discussed have presented interesting and perplexing problems. The overlapping in the clinical aspects and pathol-

ogy of the diseases they cause have made definite diagnosis difficult and retarded recognition of infectious bronchitis and pneumoencephalitis as distinct diseases. It is of interest that no immunologically different strains of the viruses of laryngotracheitis, infectious bronchitis or pneumoencephalitis have been detected. The immunological relationship that has been shown to exist between the viruses of pneumoencephalitis and Newcastle disease is particularly difficult to interpret. Is, as some contend, pneumoencephalitis merely a benign form of Newcastle disease which at any time may take on the devastating characteristic of the latter, and if so, when and how was it introduced into the United States? Could it be that the highly fatal virus disease which caused severe losses among market and farm poultry in certain eastern states in 1924-25, and occurred again on a smaller scale in 1929, was incorrectly diagnosed as fowl plague; and that instead it was Newcastle disease, which since has lingered in a benign form rather than having been eradicated as believed? In the descriptions of this disease both respiratory and nervous symptoms are said to have been observed[60 61 62] in some of the affected birds, and no mention is made of any attempt to differentiate the virus from that of Newcastle disease. This question is raised as one possibly worthy of consideration, since thus far no explanation of the presence, in the United States, of a virus related to either Newcastle disease or fowl plague has been forthcoming.

The foregoing falls far short of covering the subject of animal viruses as was requested in the invitation to participate in this colloquium. It was thought, however, that the scope, type, and accomplishments of research on animal viruses could perhaps be more clearly shown by discussing a few virus diseases in some detail, rather than by attempting to cover all of the larger number of virus diseases of animals and giving only brief consideration to each. It was quite natural and perhaps also justifiable for the writer to select those to be discussed from the ones with which he is most familiar. Accomplishments comparable to those described have been made with respect to virus diseases of all species of farm animals. Progress in research on poultry viruses, however, may have proceeded a little faster than with virus diseases of other types of farm livestock because the natural host of poultry virus has a short life span and is inexpensive; moreover, large colonies of normal stock can easily be maintained in a disease free environment, and they provide an otherwise excellent type of experimental animal. The fact that three new virus diseases of poultry have been identified during the past 25 years suggests that others may be in the future. In fact, preliminary reports concerning four additional virus diseases have recently appeared in the literature. Knowledge gained from the past should substantially aid in solving new virus problems as they arise.

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# At the Borderline of the Living

WENDELL M. STANLEY

MR. CHAIRMAN and ladies and gentlemen, I have enjoyed my day in Corvallis very much, and I look forward to returning many times in the future. I lived in the Midwest for over 20 years, followed by a little over a year abroad in Germany and traveling on the continent, in England and Scandinavia. I returned to the United States and lived for another 17 years on the East Coast, so I think I have a pretty good idea concerning living conditions here and abroad. I am convinced that the West Coast really represents the area of the future in the United States.

We've had a rather varied and sumptuous fare today in connection with viruses. I have been to a great many symposia, and I think that this one (with perhaps no more than one or two others) was quite outstanding from the standpoint of the variety of subject matter which has been presented. I think it would be almost impossible for any of you to have listened to today's discussions without going away with a pretty good idea—a bird's eye view at least—of viruses. You've heard some very good summaries, particularly the paper given at luncheon and the final paper this afternoon, covering the poultry viruses. You also have had a good presentation of the practical problems in the cheese-making industry from the standpoint of virus diseases. You learned about a wide variety of plant viruses in the presentation by Dr. Milbrath. I was particularly interested to hear Dr. McWhorter ask for questions as to whether viruses had anything to do with neoplasms and then proceed to show that they certainly did in the cases of certain plant virus diseases. He mentioned some of the animal viruses that certainly cause cancer in animals, and I think this is a subject that must be faced in the future. Dr. Freitag presented a very interesting summary of his work and the work of other individuals in connection with insect transmission of viruses. This subject is very important because insects provide one of the major means by which viruses get around, and when viruses get around to new hosts, unfortunately, they cause trouble for us.

This evening I have taken on the task of speaking about this borderline area of living things because I think it is a subject that should interest all of us. It concerns the nature of life itself. You recognize your neighbors as living organisms, but have you ever taken the time to wonder about the real difference between something that we ordinarily regard as a living entity and the metal of the microphone here, or the metal in your knife and fork, or the salt or sugar that you see on the table?

This problem, as I hope to develop it, will consist not only of an exposition of this fundamental question of the nature of life, but will touch upon subjects which interest us from other points of view. It will concern the nature of cancer, not only in animals, but projected to the problem in man, the basis of infectious disease, particularly those we call the virus diseases, which have become much more prominent by virtue of man's control over most of the bacterial diseases and also, as Dr. Dornfeld indicated this noon, the question of the nature of the gene. This question of the nature of the difference between living and nonliving things is one which has been much discussed by philosophers for a great many years.

What is the basic difference between you, let us say, and a rock or a piece of metal? If you have taken the time to ponder this question, you have done something that was done at least 3,000 years ago by Aristotle and probably by others years and years before him. In other words, man has been interested in the difference between living and nonliving things for thousands of years. It is very interesting that Aristotle, as he pondered this question, came up with the suggestion that Nature makes so gradual a transition from the inanimate to the animate, or from the nonliving to the living, that the boundary lines between the two are doubtful or perhaps nonexistent. Aristotle had very little to go on in those days, other than sheer reflection—no microscopes, no electron-microscopes, very little experimental data, and yet he suggested the idea of a continuous spectrum from the living to the nonliving. This theory, involving smaller and smaller living organisms, was taken up, particularly by medical people, and one of the early physicians, Fracastoro, wrote a book based on the conception of small and still smaller living entities as the cause of infectious disease. Again he did this without experimental data at his disposal, simply made a suggestion; but, unfortunately, it was a suggestion that fell upon ears which heard not, because this entire book was forgotten. Of

course, during that time man depended only on what he could see with his own eyes in order to arrive at knowledge of the existence of these minute living organisms, for it was not until the invention of the microscope by Leeuwenhoek, in 1680, that man for the first time was enabled to see things smaller than can be seen by the naked eye. As you know, so far as man was concerned, a whole world of small living entities came into existence via Leeuwenhoek's microscope.

This development was followed almost 200 years later by experimental proof of the relationship between these small living entities that could be seen by means of the microscope and a quite wide variety of diseases of man and of animals. You remember—Dr. Strand referred to it this morning—Pasteur, Koch, Davaine, and others, working with these bacteria showed that they were responsible for infectious disease. These scientists were extremely vigorous and very successful, for disease after disease was shown to result from the activities of these little living organisms, the bacteria. These bacteriologists lived through what has come to be known as the "Golden Era of Bacteriology" and, as I indicated this morning, the thinking during that period was such that, when Iwanowski made his discovery of the first virus disease in 1892, he refused to believe his own experimental data, presumably because the trend of the times and the thinking of the times was that such diseases were supposed to result from the activity of bacteria. Going down through the years, and using Leeuwenhoek's discovery, the microscope, bacteriologists reached a new borderline, that is, besides what one could see with the naked eye and with the ordinary light microscope, somewhere in the neighborhood of 200 millimicrons. That represented a new borderline. One could not see anything below that size.

Some chemists, in the meantime, working with small entities and molecules, molecules of organic materials, carbohydrates, etc., eventually got into the proteins, into larger and larger proteins, and devised means of proving that some of these larger proteins were in the neighborhood of 10 millimicrons in size. So chemists, working with very small entities, had worked up somewhere into the neighborhood of 10 to 20 millimicrons. That represented, then, the so-called "twilight zone," a zone essentially of the unknown.

About 15 to 20 years ago, it was quite the thing in discussions of viruses to suggest that they were simply still smaller ordinary living organisms. They were down in this so-called "no man's land" between 10 to 20 millimicrons and 200 to 300 millimicrons. Experimental methods were devised to cover this area as time went by, and the viruses were found to fill this gap almost completely. The first virus, as I have indicated, was that of tobacco mosaic, discovered by Iwanowski in 1892 and then rediscovered or presented in the proper manner by the Dutch botanist Beijerinck, in 1898. In that same year, the first animal virus disease, that of the foot and mouth disease of cattle, was discovered by Loeffler and Frosch. The first human virus disease was discovered in fairly rapid order in 1901 by Reed and his coworkers. I refer, of course, to the virus of yellow fever.

Since that time, over 300 different viruses have been discovered and more continue to be discovered. A great many of these 300 viruses exist in the form of a variety of strains. The one I know best is tobacco mosaic and, so far as I can see, there is almost an endless number of strains of this particular virus. Plant pathologists have been able to pick out at least 50 or 60 strains. So if you assume there may be as many as 5 or 10 strains, on the average, of a virus, and you have at least 300 virus diseases, you can see that we are dealing with a rather widespread type of infection.

A chart of sizes of viruses and other entities can be drawn ranging from red blood cells about 7,500 millimicrons in size at the top down to the molecule of egg albumin. The pleuro pneumonia organisms are listed as 150 millimicrons. They represent the smallest of the accepted living organisms. They cause a pneumonia disease in cattle. Somewhat larger are the brick-bat-shaped entities that represent vaccinia virus or the pox viruses that Dr. Beach mentioned this afternoon. That, incidentally, is the material that is used to vaccinate or protect you against smallpox. In a chart of this type there is an overlapping in which some viruses are larger than certain accepted living organisms and, at the bottom of he scale, one finds quite a number of important viruses, tobacco ring spot, Japanese encephalitis, alfalfa mosaic, tobacco necrosis, and foot-and-mouth disease of cattle which are smaller than certain protein molecules. Those of you who were interested in Dr. Dornfeld's paper this noon will be interested in the fact that on such a scale the best estimates of the maximum size of the genes would place them near the middle of the viruses. There is new evidence that indicates this certainly is a maximum size and that genes are probably considerably smaller.

Pictures painted in 1619 show tulips having the sort of variegation which interests Dr. McWhorter particularly. It appears that we had these plant viruses hundreds of years ago, and there is reason to believe that animal viruses and other human viruses were in existence literally hundreds and hundreds of years ago.

As I indicated this morning, in order to get some idea of the nature of these viruses, it was necessary to select one of them and subject it to a detailed study. This was done, beginning about 1932. Using the ordinary methods of protein chemistry, the crystals were obtained of a material that turned out to be a high molecular weight nucleo-protein. The individual particles or molecules of this nucleo-protein are 15 by 300 millimicrons in size.

The particles of the tomato bushy stump virus, instead of being rod-like particles, are spherical particles about 40 millimicrons in diameter. I would like to show a picture of the crystals of this virus at this particular time because, if you will refresh your minds, at the moment we are talking about living things and nonliving things, and about some of the outstanding characteristics of viruses—their small size, their ability to grow or reproduce when within the living cells of certain specific hosts, and their ability to change or mutate during their growth or reproduction within those living cells. Of course, the idea of specificity is present in that certain viruses will grow only within certain kinds of living cells. You will recognize the property of growth or reproduction and this property of change or adaptation or mutation, as properties which have long been described as belonging to living entities; yet the spherical particles which can form the crystals possess those properties. You may still find, as I do, that it is a little bit difficult to think of this in terms of a living entity—a metabolizing entity. Yet that really represents the problem one is facing, because a single one of those little particles which go to make up the crystal, when introduced into a susceptible plant, is either duplicated or reproduced so that literally millions of similar particles appear. Of course, this process of reproduction is one which, in the past, we have always ascribed to living entities.

To point up this unusual property of mutation, I should like to describe what happens to a plant when a virus mutates. If one searches the leaves of mosaic-diseased Turkish tobacco plants one will find a yellow spot on a few leaves. There, essentially before your eyes, you have the evidence of a mutation. If you isolate the virus from that spot, you will find that it is not like ordinary tobacco mosaic virus, but that now it is a yellow mottling virus. It is different—the disease which is produced by the virus from that yellow spot is different. So something has happened—we call it a mutation. The plant pathologist is extremely adept at isolating viruses from spots such as that or from the so-called local lesions which are produced in certain plants. He has been able to isolate a wide variety of so-called strains of viruses by this means.

One of these is a virus strain isolated by Dr. Jensen, known as a J-14-D-1. It causes local lesions of a special kind. Another interesting virus strain is known as the Holmes' rib grass virus. It seems to be a strain of tobacco mosaic. These two are, to the plant pathologist, just as different from one another as night from day. In general, the diseases they cause are quite distinguishable from the ordinary tobacco mosaic disease.

The ordinary tobacco mosaic, when applied to the Turkish tobacco plant causes the plant to be mottled and stunted a bit, but it moves on and flowers and produces viable seed. However, when the J-14-D-1 strain of virus, which presumably was derived from tobacco mosaic, by virtue of one or more mutations, is introduced it kills 100 per cent of the Turkish tobacco plants into which it is placed—an invariable result. If you need a bit of mental stimulation, you might think of this in terms of some virus such as poliomyelitis. The change which takes place in the ordinary strain of poliomyelitis virus, which normally goes through communities as a relatively benign virus, into the paralyzing or killing type of poliomyelitis which springs up now and then, could be just like this so far as principles are concerned.

What is the nature of the difference between the strain which seems to get along with its host and the strain which kills its host? If you think of the host-virus relationship, you will see why strains such as this do not get along well in nature. Obviously, if you are so rude as to kill your host every time you are invited out, you will soon run out of hosts; consequently, as with the viruses, if you are dependent upon hosts, you have no one left to feed you. Therefore, you end up in the same way as your host. In other words, you cannot survive either. So strains such as this have to be kept going by artificial means such as in a glass-house by man. These strains, as I have already indicated, do not get along well in nature. It is the strain which is

well adapted to its host so that they both will go on symbiotically that survives. But, of course, if a sudden mutation should take place in the virus affecting human beings, and be extremely injurious, in the situation portrayed in George Stuart's book, "Life Abides," it will not do us much good to know that—if such a virus should arise and wipe out the entire human population—the virus itself would eventually disappear, because we at least would have gone first. From a fundamental standpoint, what is the nature of the change in the virus which suddenly permits it to become a killing type of virus? That is a most important problem, and it is one that has interested us very much.

Being chemists, and knowing that the viruses had different biological activities, we knew that there had to be a difference in the structures somewhere along the line. We, therefore, decided to take a long shot. Since these are nucleoproteins, we examined the nucleic acids which represent one component. So far as we could tell, the nucleic acids were identical. The only thing that was left to examine were the protein components. The proteins are made up of amino acids. Now was it possible that there was a difference in the amino acids, or would this new property of killing simply depend upon some minute change in the order of amino acids? Having got little or nowhere with other approaches, we thought the very least we could do would be to have a go at this, despite the fact that it involved a tremendous amount of work.

Amino acid analyses of these purified preparations of a variety of strains of tobacco mosaic showed that there were significant differences between strains. In some cases the differences are very great, as, for example, the fact that some strains contain no cystine—a complete absence of that amino acid. In the case of the Holmes' rib grass virus, a whole variety of changes in the amino acid composition has occurred. One fact that interested us is that in the case of one strain there are really only two changes. The plant pathologist has some reason to suspect that this virus strain arose as a result of two mutations. Could it be that one mutation occurs when some change in one particular amino acid takes place, and that the new strain goes on a bit and mutates again, accompanied by this change in the second amino acid? These are things that are unanswered at the moment.

If Nature can do things like this—if Nature can introduce new amino acids—to take amino acids out of the virus structure, how about us as chemists? Would it be possible for us, as chemists, to make changes in the structures of the viruses, put them back into the host and get a new type of disease? Well, we asked ourselves that question. You see the potentialities there. In the virus, you have a chemical structure that you can tinker with in the test tube, and then you put it back into a living cell, resulting in the reproduction—many times over—of the structure. You have the possibility, at least, of chemical tinkering with essentially the germ plasm of life itself. If you are a bit of a philosopher, you might just carry that trend of thought to its ultimate conclusion, and you see what you can do with the world. It is very interesting.

As chemists, we decided to have a go at this. Being a bit timid, we started with just tiny changes because we did not know what we might get into. Actually, we introduced somewhere in the neighborhood of 5,000 chemical groupings around the periphery of this molecule of tobacco mosaic virus. The fact that they are in, and that certain amino groups have been covered, is proved because the electrophoretic mobility has been changed. In other words, if you cover up an amino group, which is a basic group, with an acetyl group, you essentially block the basic properties engendered by that amino group; consequently, they will move at different speeds in an electrophoretic field. If you take a mixture of treated and untreated virus, for example, and subject the mixture to electrophoresis, you find them separating out again. In other words, you can prepare your derivative and then, to prove that it is different, you mix it back with what you started and then subject it to electrophoresis to show that you can separate the two.

Now, of course, the important question and one which may be entering your minds already is, what happens or what did happen when you put these chemical derivatives of this virus back into a host. The first thing that happened, one that interested us very much, was that we knew we had already made a change of sufficient magnitude so that the biological activity was changed. Well, that was one step. The next thing, of course, was to infect such plants, isolate the progeny from the inoculum and determine whether or not this had reproduced true to form. So far, unfortunately, it has not. We introduce an acetyl derivative or a benzene sulfonyl derivative of the virus and, although it goes in with either greater or lesser difficulty indicating a change in biological activity, the virus that is produced as a result of that inoculum is the ordinary strain of virus

once again. That tells you right away that the changes that we made are not the changes in the structure which have to do with this reproductive mechanism. We are now engaged in making still greater changes in the structure. We are now introducing, as Nature did, new amino acids into the virus structure; that work is in progress and perhaps later on I shall be able to report on that to you. I have, however, every reason to suspect that we can duplicate what Nature has done and, by so doing, it may be possible to secure virus vaccines at least of a kind which will be useful for mankind. It is possible, in other words, that your chances of getting something that is benign are just as good as your chances of getting something that is very virulent. And since these viruses are produced in the laboratory under controlled conditions, you simply keep your virulent strains bottled up and go ahead and work with the others.

Figure 5 is essentially a full story in pictures. It contains the essence of what I have been attempting to tell you tonight. It simply is a series of electron micrographs of purified preparations of viruses, ranging from the large elementary bodies of vaccinia in the upper left, down to small spherical particles of the tomato bushy stump virus in the lower right hand corner. As they

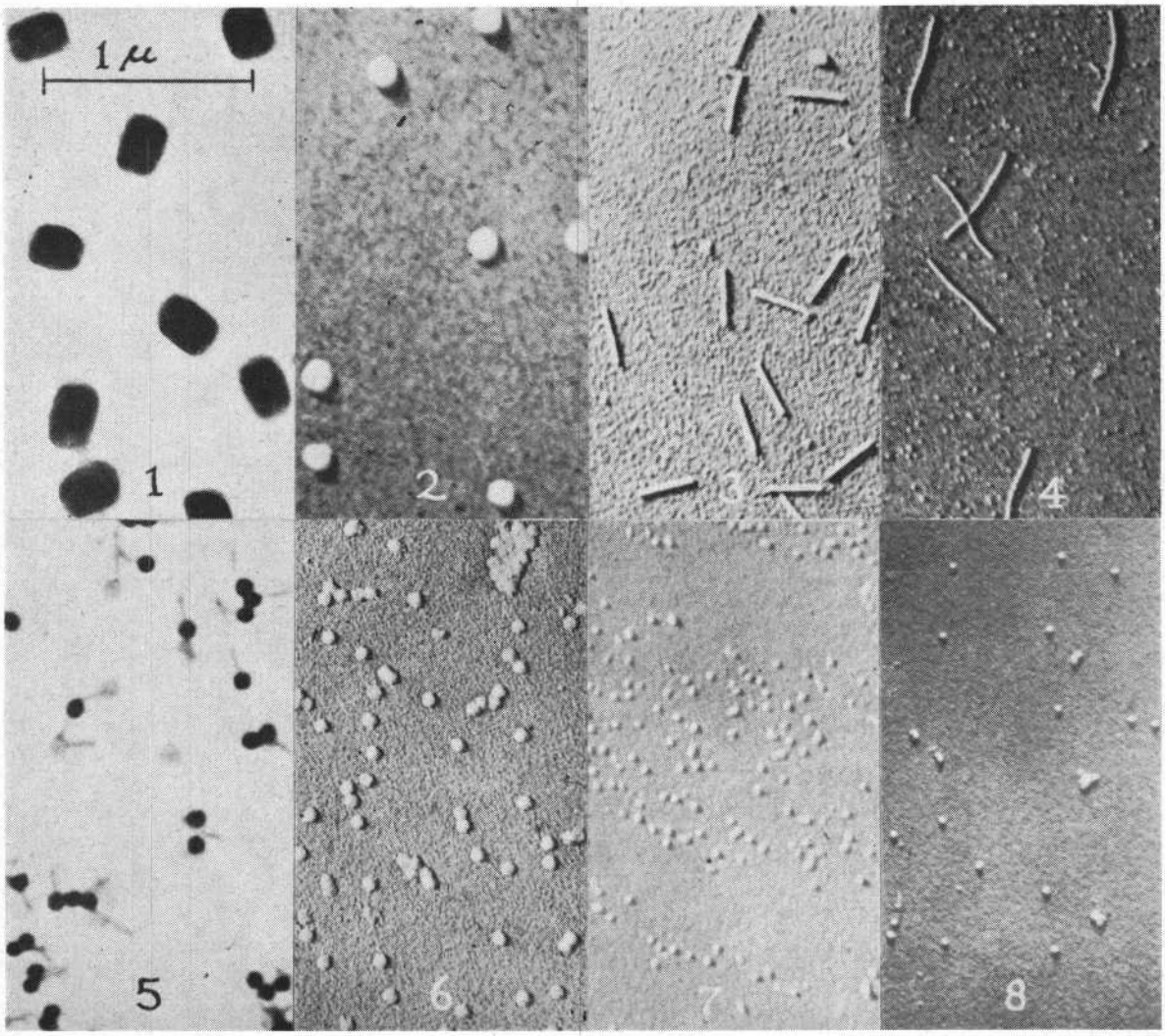


***Figure 5. Electron micrographs of purified virus preparations. 1. Vaccinia virus. 2. Influenza virus (Lee strain). 3. Tobacco mosaic virus prepared from hair cells. 4. Potato X virus (latent mosaic of potato), hair cell preparation. 5. $T_2$ coli bacteriophage. 6. Shope rabbit papilloma virus. 7. Southern bean mosaic virus. 8. Tomato bushy stunt virus.*** *Used by permission of American Chemical Society*

are all at the same magnification, the differences you see are real. Now, as I proceed, you might begin to think about the point, if any, at which life begins. In the upper left hand corner are the elementary bodies of vaccinia which are brick-bat shaped particles, about 300 millimicrons in size. These represent one of the very early human diseases. Smallpox has been known for thousands of years. As a matter of fact, a strain of this virus represented the first successful vaccine. Even before the discovery of viruses, Jenner noticed that if he took a little material from the cowpox lesions on a cow and applied them to a scarified area on the human being, he would get an immunity against smallpox. Even before this time, it was known that you could take the dried material from individuals who had come down with smallpox and apply it to normal individuals. While many times you would get frank smallpox, a certain percentage of the time you would get an abortive reaction or local lesion type of reaction followed by immunity. The next one (number 2) represents particles of influenza virus, which look like nice, puffy, very pretty little balls, about 120 millimicrons in size. That virus turned out to be the greatest killer of all time. Before it was known to be a virus disease, the influenza virus, as you know, went on a rampage in the fall of 1918 and spread practically all over the world. That winter, approximately 500,000,000 people had influenza and of these, about 15,000,000 died. Here in the United States, we lost through the activity of that little white puffy material over 400,000 people within four months. To place that in proper perspective, I shall ask you to remember our total casualties on the battlefields of World War I plus World War II, the greatest of our world wars. Our total battlefield casualties in both those great wars comes to about 400,000 men and women. Here was one virus disease which, in four months during the winter of 1918, killed as many people as lost their lives in this country in two great world wars. It really is an amazing situation, because in 1918 the cause of that epidemic was unknown. You would have thought that, in the case of an attack of that sort—with a secret enemy, so to speak, attacking from within—Congress would at least have held an investigation; but it did not. Other than the efforts made by less than a dozen men working on this disease throughout the world, nothing was done. It seems utterly fantastic, yet that is the case. It was not until 1931 when Dr. Shope, a good friend of mine, working at the Rockefeller Institute at Princeton, discovered for the first time that a common disease in swine in the Midwest was due to a virus. This was followed, in the work of Andrewes and Laidlaw at the National Institute for Medical Research in London in 1932, by evidence that a similar virus in man was responsible for influenza. So that is the story of influenza in those early days.

Viruses were finally discovered, first in swine and then in man, in 1931 and 1932. Despite those discoveries in 1931 and 1932, there was no effective vaccine against influenza when we entered the recent war. So far as protective measures were concerned, if there had been an outbreak of influenza at home or among our troops, during the first $2\frac{1}{2}$ years of the war, we should have been essentially helpless except for the fact we could have controlled the after-effects or secondary invaders much better than we were able to do in 1918. One of the tasks that was assigned to our laboratory at Princeton was the production of an effective vaccine against influenza. We had been working on plant viruses, of course, up to that time; and almost overnight we dropped the greater part of our plant virus work and took on this task. A successful vaccine that you can now buy in the drug stores and which is called the centrifuge-type influenza vaccine, was the result. It has been successful except for the winter before last, when many people who had been vaccinated went merrily ahead and came down with influenza. It turned out that, winter before last, a new strain of the virus had appeared; and the strains in the vaccine would not protect against this new strain in human beings. That strain, fortunately, was isolated fairly rapidly and incorporated into the vaccine. Ever since everything has been all right. In the meantime, "collection agencies" have been set up throughout the world, so that any influenza-like strain which appears anywhere in the world is flown to New York, Washington, and London, and typed, so that I think we shall not be caught in a similar predicament in the future.

The story of tobacco mosaic virus, which is Number 3, has been told fairly completely. In view of some of the suggestions made by Dr. McWhorter, I might discuss Number 4, which is properly named the "X Virus." As you can see, it has an "X" formed by the rods. It is also called the healthy potato virus. Dr. Milbrath said this morning that all the potato plants in this country contain a virus. Well, this is the virus, the rod-like things you see there in Number 4, that is present in practically all potato plants grown in this country. As he indicated, if you

make an extract out of the ordinary potato plant and apply it to another so-called healthy potato plant, nothing happens, because the virus is already present. He showed how, in this particular case, if you apply that extract to another type of a host, you can get an indication of a disease symptom. Yet think for a moment what your position would be if you did not have this second host. Suppose you were limited to a situation in which you had to prove the existence of an infectious agent, and you had at your disposal only hosts or test animals already containing that agent. I hope you see that it would be extremely difficult, if not impossible, to prove the existence of this infectious agent. Tie this up with what Professor McWhorter said about cancer. Suppose—just suppose—that human cancer is caused by a virus, and that it is essentially ubiquitous. You have no individuals who do not have a strain of that particular virus. How, then, can you ever demonstrate the infectious nature of that particular entity if no other host will receive it and allow it to grow? I do not say that that is the situation, but I do say that is the situation with the healthy potato virus if you assume that it would not go to any second host. Now how does this arise? Does it represent a normal constituent of some other plant, which in the dim past appeared in the potato plant? Does it represent some antigenic material normally present in potatoes which, at some stage of the game, got loose from the chromosomes and went merrily on its way reproducing as a separate unit? These are very interesting questions.

You saw, this morning, some of the electron micrographs of the bacteriophages. Number 5 in Figure 5 is one of the few here which is not shadow-cast. It is in its natural condition and, as such, shows up some of the things which were not shown this morning. I refer to these ghosts, in which the inner material has apparently spewed out, leaving a membrane. If you contrast this with Number 8 which forms the crystals, you see right away the sort of thing which represents quite different places on this spectrum as we go from living things to nonliving things. You have here a type of morphological differentiation that I, as a chemist, would not want to throw into the molecular world. I would certainly say that this was more organism-like than it is molecule-like; yet it has the same basic properties that you find in other viruses.

Number 6, the rabbit papilloma virus, is a very interesting virus. It was discovered by Dr. Shope when he was hunting one winter in Iowa. He noticed that one of the dead rabbits had a little wart on its under side, so he cut it off and took it back to the laboratory. There he ground it up, and applied the material to other rabbits and found that it would cause these warts or papillomas. He gave a little bit of the virus to Dr. Rous at the Rockefeller Institute in New York. Rous put it on some ordinary domestic rabbits, as Shope was also doing, and they both noticed that when they grew warts or papillomas in domestic rabbits they were not infectious. They went back, got some wild rabbits from Iowa, and tried it again. They produced papillomas and these, when ground up, were infectious. When applied to other wild rabbits they did cause warts. This was rather interesting but, even more surprising, when these domestic rabbits containing the warts were held for an additional month or so, Dr. Rous noticed that the warts invariably progressed and became cancers. Still they could not find the virus. Yet they found later if they ground up these so-called noninfectious areas, either warts or this cancerous tissue, and used that matter as an antigen, they could produce neutralizing antibodies directed against the virus, which means that the virus must have been there all the time. There is a very interesting experimental problem in connection with what happens when you put this virus into the domestic rabbit and the virus seems to disappear but then you get cancer from it. That is a problem we are still working on, not only because it is a very nice virus to work with but because of the importance of working out what happens when this virus disappears. We have had some experience with virus inhibitors and all that sort of thing; and there is an explanation for it, which will out sooner or later. But that again, you see, enters into the general picture of the relationship between viruses and neoplasms, which Dr. McWhorter talked about earlier.

Number 8 in Figure 5 is the tomato bushy stunt virus, which gives quite beautiful crystals. Now, if you are really interested in placing a dividing line between living things and nonliving things, you have the opportunity to draw a line somewhere near this virus. For myself, I choose to stand with Aristotle. I think that the philosopher Aristotle made just as good a suggestion 3,000 years ago regarding the nature of the difference between living things and nonliving things as it is possible for me to make with all the wealth of information from about fifteen years of work on viruses. So far as I can see, there is a continuous spectrum as one goes from frankly living things

down to frankly nonliving things. You find people arguing that what they call viruses are simply examples of what is called retrograde evolution. They cite examples of living organisms which, when symbiotically attached to other organisms, gradually lose the ability to perform certain functions; and they will carry that on down until they end up with what they call "naked" viruses, which can do nothing but reproduce under certain very special conditions. They have lost all other of their morphological characteristics except that final kernel of nucleo-protein which makes them a reproducing unit. Other people like to think that viruses arose and represent the beginning of what we call "life." An entire book has been written on the subject in which one starts out with essentially inorganic material and, from there, through the use of lightning flashes and heat, etc., one builds up carbon compounds and then carbon-nitrogen compounds. After that the proteins are produced and then occurs the gradual formation of the self-duplicating mechanisms.

Dr. Horowitz and Dr. Beadle of the California Institute of Technology, using the results of their work with neurospora, have presented a similar line of thinking. But regardless of the origin of living things, we are still faced with facts as we see them today. It seems to me that we can think of the difference between living and nonliving things much as the chemist has worked out the problem in the case of acids and bases, or sour and sweet. He took pH 7, arbitrarily, as a line of division. Things more alkaline go on one side; and things more acid go on the other side; and the difference between sour and sweet, so far as the chemist is concerned, depends upon which side of pH 7 you are on. Of course, we could do the same thing with viruses. We could simply have a man-made arbitrary line of division, but I do not see that this gets one anywhere. It is no more than attempting to decide the difference between black and white. As long as something is black enough, or as long as it is white enough, you can say that it is black or white; but when you get into the grays, then you have difficulty. It is the same way with the viruses. As long as they are big enough and tantalizing enough, you think they are alive; but when you get down and talk about the tomato bushy stunt virus and the crystals there, so far as the chemist is concerned, I feel no compunction and see no difficulty whatsoever in regarding tomato bushy stunt virus as a plain, ordinary chemical—a nucleo-protein chemical—and yet I cannot talk in such terms about vaccinia or some of the larger viruses. You hear once in a while the suggestion that the whole is always equal to more than the sum of its parts. When I hear an argument like that, I like to go back again to chemical structure and ask you to think just for a moment about a very simple chemical—one that you are all familiar with, i.e., the water molecule. You may not be familiar with the water molecule, but you are certainly familiar with a glass of water. Those of you who are chemists, and those of you who are not, know that water is made up of hydrogen and oxygen. I would challenge chemist and nonchemist alike to predict the properties of the water molecule, knowing the properties of the two gases that make it up. In other words, even in a very simple structure such as the water molecule, $H_2O$, you immediately get the characteristic chemical structure, two atoms of hydrogen and one of oxygen; you have something new. You have a new kind of chemical structure and with that you have the opportunity for expressions of that structure. Now, I think that you can compare that type of thinking to the viruses. When you get a sufficiently complex structure, there is the possibility of more and more complex expressions of that structure so that self-duplication, within certain media and certain living cells, can be regarded as an example of what can be expected—logically, I think—as you get more and more complex chemical structures assembled in entities such as we now know the viruses to be.