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Lecture #47 - Virus Genetics: Plant and Animal Viruses
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The viruses that I plan to discuss in this session have perhaps greater topical interest than the ones attacking bacteria which were the subjects of previous lectures, bacteriophage. The main utility of bacteriophage seems to be providing experimental material for genetic analysis, previous attempts to use these particles for therapeutic purposes having, for example, failed so far. There are, however, a number of types of viruses which attack animals on the one hand and plants on the other, and these are of great economic and medical significance. Unfortunately, in many respects, technical difficulties have hindered the collection of precise quantitative information along directions that have been indicated in bacteriophage research.

One of the chief difficulties in dealing with the particles of viruses attacking higher organisms, and especially those attacking higher plants, is the problem of accurate quantitation of the number of infective units in a sample. For example, in order to titrate the quantity of virus attacking the leaves of a higher plant, it's necessary to rub a sample of the virus over the surface of the leaf, with the certain knowledge that only a small fraction of the virus particles that might be present in that sample will find susceptible cells in the leaf surface and be capable of penetrating them to give what, in some cases, appears as a demonstrable lesion. Similarly, if one is dealing with an animal virus, such as poliomyelitis, under circumstances requiring the use of intact animals as the host for the propagation of this virus, the accurate determination of the number of particles is likely to be an extremely expensive and time consuming process. Some mitigation of this difficulty has been achieved in more recent years by

the development of plating techniques for the production of plaques of animals viruses, quite similar to the clearing plaques that were demonstrated for bacteriophage. For example, it is possible to achieve an accurate titration of poliomyelitis virus by plating samples onto agar layers which have been seeded with cells from tissue culture susceptible to this virus. And great progress has been made during the past very few years in the genetics of polio virus using techniques of this kind.

Unfortunately, for many other viruses on which one would like to get more precise genetic information there is not yet a sufficient cytopathic effect of the virus on the cells which are attacked by the virus to obtain detectable clearings on agar plates of this sort. And for these one must still use much more laborious techniques of limit dilution. For example, the influenza virus can be adapted to grow in the fluids, or rather on the cells lining the fluid cavities of the chick embryo. It is possible to obtain a titration of the number of influenza virus particles present in a given sample by diluting that sample and innoculating small aliquots into each of a series of eggs, and then after 48 hours, or some later time, harvesting the eggs and determining what fraction of the eggs did contain a virus particle. Provided one is working at near-limit dilutions, that is to say at dilutions where the probability that any one sample will contain a virus particle is low, it is possible to estimate the content of virus by the fraction of eggs which yield detectable virus.

The same procedure can be used for the isolation of clones. That is to say, under conditions of near-limit dilution of the sample, those eggs which do contain virus are at least likely to contain a population of virus that had descended from a single particle, and most of the work on the genetics of the influenza virus, which has been the most extensively studied from a genetic point of view, has been done on this basis.

Before taking up the specific questions of genetic recombination, I would like to survey some of the general features of the life cycle of some animal, and to some extent these are paralleled by plant, viruses, although one must admit in the first place that there is a much wider variation in the characteristic behavior of the group of viruses indicated here, and second that our detailed knowledge is somewhat scant in many respects.

One of the difficulties in dealing with the mammalian cell is that its boundaries are to some extent less well defined than are the boundaries of a bacterial cell. The bacterial cell in many cases has a very well defined rigid coat, which is responsible for the characteristic shape that one observes in such cells. The mammalian cell is a much more flexible object, its shape often determined by exterior forces acting on it at the time, and one now knows that there is some ambiguity as to the precise margin as to the mammalian cell -- where it ends and where the medium in which the cell is immersed begins. In fact, the outermost layer of the cell consists of a mucoid coat which acts as a substrate for an enzyme found on the surface of the virus and therefore constitutes a receptor for that virus. The structure of the influenza virus can be symbolized as an outer coat on which this virus-destroying and virus-reacting enzyme is found, and an inner material consisting of ribonucleic acid which corresponds to the genetic material of the bacteriophage.

The first step of virus adsorption to the mammalian cell is presumably the attachment by means of the superficial enzyme particles to the mucoid coat, followed eventually by the penetration of the cell by the virus particle. The details of this penetration are not well understood, it has been suggested that it may consist simply of the engulfment of the virus particle by the normal pseudopodial activity of the cell to which

it is attached. It is a necessary condition for attachment of the virus that the mucoid coat be intact, as can be demonstrated by stripping the coat by means of specific enzymes or reacting substances such as periodate. Once inside the cell the virus particle enters an eclipse phase similar to that already described for the bacteriophage, although less is known at the present time concerning the physical and chemical basis of this alteration from an infective particle to the vegetative stage. During the subsequent several hours of growth of the vegetative virus inside othe cell there is an increase of the ribonucleic acid component, and this is followed by the gradual liberation from the cell, over a period of considerable time, of intact particles. In the case of the influenza virus there is some evidence that the virus particle does not receive its final coat except in the act of emergence from the cell from which it is being liberated -- in other words, that the coat of the influenza virus particle consists in part of cell-specific materials already present in the host cell, in part of the materials elaborated in the host, as a joint action of host genetic materials and the genetic material of the virus itself. Whether the details of this picture can be applied equally well to other viruses is not at all clear, and certain it is that there are considerable variations in the details with which these must be applied.

We are now in a position to discuss some explicit experiments that have been conducted originally by Burnet in Melbourne, and subsequently by investigators in this country and in Great Britain, on the possibility of genetic recombination among strains of the influenza virus. In order to accomplish this it was first necessary to secure a number of useful markers, and this has not yet been done in any very efficient way through procedures of spontaneous mutation, although this has perhaps not yet been fully exploited. Instead, Burnet has used a pair of strains designated MEL for Mel-

bourne and WSE, an egg-adapted strain, which pair of strains differ in respect of a number of markers. We may indicate here a serological difference, the marker A as against W; sensitivity to ovomucin, that is the capacity of the mucin found in egg to inactivate the virus; and thirdly the pathogenicity of the virus for the egg when deposited on the chorio-allantoic membrane. In this case the small e refers to the pathogenicity for the egg, the large E the non-pathogenicity characteristic of the MEL strain.

When cells of the egg membranes are infected at high multiplicity of infection with mixtures of these two viruses, so as to insure that many cells will have received an input of at least one virus particle of each kind, a number of interesting events have been detected in the output from such mixedly infected cells.

Before proceeding to the question of genetic recombination it's necessary to stress a somewhat different process which has been found to take place rather independently of exchange of genetic material. This is phenotypic mixing, which refers to the fact that although two quite distinct genomes are represented in the individual particles which issue from the doubly infected cell, that these particles show a common neutralizability by antiserum. In fact, in this case we have an anomalous neutralization where it proves to be the case, that the progeny from a mixedly infected cell can be neutralized almost perfectly efficiently either by the anti-WSE, or by the anti-MEL serum. This is interpreted as representing the presence on the coats of both types of virus particles, on the coats of virus particles containing either WSE or MEL genomes, of substances which will react with the antibody which reaction results in the neutralization of the virus particle. However, most of the virus particles which issue from

such crosses retain their original individuality, or at least the intact individuality of a WSE or of a MEL particle, and this phenomenon should not be confused with genetic recombination.

A second finding which Burnet has reported and which is based on the procedure for isolation of clones of virus by the method of limit-dilution, is the occurrence of particles that appear to be heterozygous. That is to say, what are nominally single clones are found to contain mixtures of two genetic types, and this phenomenon is in principle at least quite independent of the mixing of their phenotypes. The exact genetic basis of heterozygosis is debatable. One could argue that it merely represents the close adhesion of different particles to one another, which would then behave as a unit on limit-dilution. Or possibly within a single particle there are two separate genomes which can then be distributed to different progeny in further replication of the virus RNA. At any rate, this again does not yet represent a complete phenomenon of genetic recombination. There has been no exchange of parts of the genetic material of either one or the other in the formation of any stable clone of virus. And the same results as we attribute to heterozygosis would pertain, if in fact the infected fluids which we consider to be single clones had in fact derived from more than one virus particle.

Finally, among the yields of such mixedly infected cells one does find a number of particles which can yield pure clones which can be attributed to be recombinants. These are clones which will have stably the characters, say large A and small c, or small a -- I used the symbol W for that, for the alternative serology -- and large C. These recombinants are stable by the criterion of repeated single clone isolation through limited dilution and also by the virtue of the fact that although virtually all

of the virus population of this type of recombinant can be wiped out by the use of anti-W serum there is not then found a residue of the alternative type. This is just one of a number of lines of evidence for the purity of these clones, although we see already the technical difficulties which beset this type of investigation as compared to systems where we can isolate clones by simple methods of plating, as of bacteria or of plaquing or plaque formation as for bacteriophages.

Similar phenomena of genetic recombination have been found in the rather more complex virus of the vaccinia group, which Fenner has investigated, and for which a number of markers, for example the formation of hemagglutinins, resistance to heat, and specific virulence for rabbit and mouse hosts, as well as the characteristic formation of pocks on the chorio-allantoic membrane have been used as markers. At the present time vaccinia appears to be perhaps the best system in which we now have the technical possibility of investigating genetic recombination among viruses, but the results so far reported in this system have been very recent indeed.

At the present time, the virus in whose genetics there is perhaps the most practical interest is undoubtedly the polio virus, or the group of polio viruses. Here there are active efforts continually being pursued and tested on a very large scale indeed to obtain mutants of the polio virus which would be capable of acting as efficient immunizing agents and yet free from the hazard of disease and paralysis associated with typical polio. It must be admitted that the methods so far used for the selection of such mutants have been rather empirical. They have often involved the growth of the virus in unusual hosts, or in tissue culture of cells of types not associated with the nervous system, and while there have been apparent successes in obtaining mutants better adapted, say to epithelial cells than to the nervous system, we are not yet in a

position to precisely define the genetic changes which were the basis of these changes in virulence.

Fortunately the development of methods of isolation of single plaques has at least made it possible to obtain pure clones of those mutants which have been secured, and thereby to minimize the possibility that these clones subject to test had been contaminated by admixture with particles of another genotype. Unfortunately attempts to demonstrate genetic recombination among strains of the polio virus have until now been unsuccessful. If they should succeed this would be perhaps the most productive method of obtaining recombinant genotypes which would be capable of showing the immunizing activity that we are seeking, and from which we have deleted, by recombination, the specific adaptation to the human host characteristic of these viruses.

Although genetic work with the polio virus, and likewise with other encephalitic viruses and with the tobacco mosaic virus, has thus been limited to mutational studies, they have been the basis of other equally remarkable findings in the field of genetic chemistry. Several workers have found, with each of the viruses that I have just enumerated, that it is possible to separate the protein from the nucleic acid component of these viruses. These can be done, as Schramm did, for example, by exposing the virus to aqueous phenol solution which will destroy the protein and leave the ribonucleic acid intact. Conversely, the protein of the plant tobacco mosaic viruses at least, can be removed in reasonably native condition by exposing the virus to moderate alkalinity.

Two phenomena of great interest have been found. First, that in the case of these viruses, an extract of the virus containing only the ribonucleic acid portion and lacking any demonstrable protein does retain substantial infectivity. We might con-

sider that if we were dealing with a hypothetical virus particle, which we do believe in general consists of an outer protein coat and an inner genetic component of the nucleic acid, that in fact only the enclosed RNA is essential for the maintainance of the state of infection of a cell into which it is introduced. On this basis, the primary function of the protein coat is to act as the nose cone, so to speak, for the preservation of the virus particle in transit from cell to cell, and for the reentry of this particle into a new cell which it might infect. Given this, and this is merely a restatement now of the observations that have been made, the isolated nucleic acid may be expected to be infective provided it is kept under rather gentle conditions, so as to minimize the possibility of its chemical inactivation while it is in solution, and provided there exists some mechanism for the reimportation of the virus nucleic acid into the infected cell. We do not know what that mechanism is in the case either of the tobacco mosaic virus or of the mammalian cell, but this does appear to happen, that isolated nucleic acid is capable of penetrating the mammalian cell. Once having penetrated the cell, this forms part of a vegetative pool of virus which is, in fact, identical to the normal fate of intact virus that might enter the cell, since we have reason to believe, as I will elaborate further, that the protein plays no important part in the further replication of the genetic material, or in the production of new coats for that matter.

Once inside the cell this vegetative pool enlarges, particles will then mature, and will develop whatever protein coats are specified to be around them by virtue of the information contained in the nucleic acid inside the cell, and they will then be followed by the liberation of intact virus of the usual sort at some later interval. At the present time different preparations of purified nucleic acids have infectivities ranging from a very small proportion, perhaps one part in several thousand, to (a) several

percent of the infectivity of a comparable amount of the intact virus.

The experiments which show the relative unimportance of the outer coat of the virus particle have been done by Fraenkel-Conrat of Berkeley who has succeeded in reconstituting tobacco mosaic virus by mixing preparations, on the one hand, of the nucleic acid, on the other hand, of the protein -- that these will, under certain conditions, again coalesce to form particles sessentially similar to the original virus particle. In the case of tobacco mosaic virus this is a long linear particle, and if we drew a cross section of it, would look something like this. Let this be a longitudinal section with a spiral of virus material inside a protein coat. This protein coat would be built up of many monomeric building blocks, stacked on one another in a regular array.

Fraenkel-Conrat also showed that one could make such preparations, on the one hand of the RNA, on the other the protein, from two distinct viruses, which could be distinguished from one another either serologically or by virtue of the specific symptoms on the plant material that they infected. One could, for instance, obtain preparations from a strain of tobacco mosaic virus called HR, Holmes ribgrass together with the standard tobacco mosaic virus strain. And one could now make mixed preparations which would have the protein of Holmes ribgrass and the nucleic acid of tobacco mosaic virus, or vice versa. The characteristic behavior of such particles is quite reminiscent of those which one obtains on phenotypic mixing from the issue of cells which are mixedly infected.

One should interject that this phenomenon has not been directly observed in the case of plant viruses, since in fact we have no assurance, owing to the low infectivity of these particles, that we have ever mixedly infected a single plant cell with

two viruses. This has been seen with many animal viruses.

The attributes of these mixed particles in this experiment, which is called more properly a reconstitution than a recombination experiment -- the attributes of these reconstituted particles are this -- we are dealing, let us say, with a tobacco mosaic virus RNA and a Holmes ribgrass coat. Well, such particles, unlike normal TMV particles will not be inactivated by anti-TMV anti-serum. They will, however, be inactivated by Holmes ribgrass anti-serum, since the point of attack of antibodies is the surface protein coat and has to do with the initial entry of the virus particle into the cell. However, the progeny of these particles, although the initial infecting particle was a Holmes ribgrass with respect to its protein, are all typical TMV particles. The protein manufactured by cells infected with such a particle is determined exclusively by the nucleic acid, and corresponding results were obtained on the other side with the opposite combination. From this we can conclude with reasonable assurance that the nucleic acid is the primary determinant, in fact perhaps the sole determinant, of the specificity of the proteins produced in an infected cell.

There are other factors which distinguish the stability of intact virus particles from RNA. The isolated nucleic acids are very much more susceptible to such enzymes as ribonuclease, to thermal degradation, to changes of pH. On the other hand, they prove to be even more resistant than the isolated, than the intact virus particle, to detergents and other materials whose primary effect is on the protein.

Since there are now available a number of systems where it is possible to utilize nucleic acids as primers for the synthesis of increased amounts of these nucleic acids, systems of this sort will eventually play an extremely important role in the replication of biologically active nucleic acids. At the present time we have strong evidence for

specific replication in an enzymatic system of DNA materials, but not yet for RNA materials. To this end it is therefore encouraging that there are some recent findings which support the possibility of the infectivity of the isolated nucleic acid of bacteriophages -- in this case this is DNA. These preparations consist of suspensions of bacteriophage which have been treated in somewhat the same fashion as the particles of the RNA viruses that I've indicated here. In other experiments they have been exposed to high concentrations of urea, which seems to have the effect of at least opening up the virus particles.

These preparations require to be resolved considerably further before they can be available for the same kind of reconstitution experiment as has been described for the tobacco mosaic virus. At the present time the specific replication of virus genetic material from animal and plants viruses constitutes the best explicit evidence for the exact replication of RNA, evidence which is already more than ample for DNA. Experiments on the recombination in influenza show that RNA is also susceptible to the same fundamental processes of crossingover and genetic recombination. They therefore furnish a strong motivation for searching for comparable mechanisms of in vitro replication as have been described in DNA.

Throughout this discussion the common thread has been the continuity of genetic information through the agency of the nucleic acids. However, it should be stressed that DNA and RNA are not unique materials. They have the same relationship to the information contained in them as carbon black does to the words in a dictionary.