THE DEVELOPMENT OF THE VIRUS CONCEPT AS REFLECTED IN CORPORA OF STUDIES ON INDIVIDUAL PATHOGENS*

1. BEGINNINGS AT THE TURN OF THE CENTURY**

by

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DURING THE last quarter of the nineteenth century bacteriology emerged as a separate discipline. A number of pathogenic bacteria were isolated and described, and it proved possible to grow them in culture on artifical media. At the same time, there were diseases which were being studied in great detail, but for which no infectious agent could be found.

One such example was rabies. Although the number of victims was limited, its manifestations and manner of spread were striking and caused much public concern. When Pasteur achieved the development of a vaccine, and saved the life of a young boy who would otherwise certainly have succumbed to the disease, the emotional impact and the professional response were considerable. Yet, in spite of much painstaking work, Pasteur failed to isolate a causal organism. All the evidence suggests that although Pasteur may have conceived of a causal agent so small that it eluded detection by the light microscope, he did not suspect that it differed essentially from the pathogenic micro-organisms so far observed.

Then, in the last decade of the century, work on two very different conditions yielded unexpected results. In 1892, Ivanovski, a young Russian botanist, published his report[1] of a study of the mosaic disease of the tobacco plant, carried out on tobacco plantations in different parts of Russia, and in the laboratories of the Academy of Sciences in St. Petersburg. Ivanovski found that when he passed the sap of infected tobacco plants through bacteria-proof filters, the clear filtrate remained infectious when inoculated into healthy plants. This did not surprise him, since he suspected the presence of a bacterial toxin and saw the result as support for his theory. The Russian journal in which the paper was published does not appear to have had a wide distribution outside Russia at the time,[2] and Ivanovski's results passed largely unnoticed.

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**The origins and the early development of the virus concept are described in comprehensive detail in S. S. Hughes, "The origins and development of the concept of the virus in the late nineteenth century", thesis, London University, 1972; and in S. S. Hughes, "The concept of the virus: a history", *in press*.

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But, a few years later, the Dutch botanical microbiologist Beijerinck turned his attention to the same subject. For Holland as for Russia, tobacco was of economic importance, and much of the early work on diseases of the tobacco plant was done in Dutch laboratories. Beijerinck was unaware of Ivanovski's work, although he immediately acknowledged Ivanovski's priority in showing the filterability of the virus when he learned of it. But when his own paper was published, in Dutch in 1898,[3] and in German[4] the following year, it was obvious that his approach had been different and that he had collected experimental evidence which lent itself to a rather more extensive interpretation. Beijerinck also passed the sap of mosaic-diseased plants through a porcelain filter and found that it remained virulent; but instead of inoculating only one set of plants as Ivanovski had done, he showed that the mosaic disease could be transmitted in series, which allowed him to conclude not only that the agent passed through the "bacteria-proof" filter, but also that it apparently was able to multiply in the plants. When he had gathered all of his experimental evidence, including data from diffusion experiments, it was clear to Beijerinck that he was dealing with an infectious agent which was essentially different from the cellular micro-organisms hitherto isolated and identified as pathogens.

Beijerinck reached his final conclusion almost reluctantly, and presented it with some diffidence; in retrospect it is astonishing that he was able to form such a concept at all at the time. For, having concluded that the virus must be present in the form of dissolved molecules, he went on to propose a manner of replication which would account for the fact that the virus appeared to multiply only within actively growing tissues, viz., that "the contagium, in order to reproduce, must be incorporated into the living protoplasm of the cell, into whose reproduction it is, in a manner of speaking, passively drawn". He explained that he had arrived at this possibility because "although the reproduction or growth of a dissolved particle is not unthinkable, it is difficult to imagine. Molecules equipped with a division mechanism enabling them to reproduce, and the idea of metabolizing molecules* which must be a presupposition, seems to me obscure if not positively unnatural".[4]

While Beijerinck was working on tobacco mosaic, a commission was set up in Germany to investigate means of combating foot-and-mouth disease, a condition presenting a serious threat to the cattle-farming industry. The commission was headed by Loeffler and Frosch, and it was their names which appeared on the reports.[5] In the course of their investigation Loeffler and Frosch passed samples of vesicular lymph from infected cattle through bacteria-proof filters in a search for possible dissolved immunizing substances. The calves inoculated with the filtered lymph did not acquire immunity; they developed the disease with all its typical symptoms after the usual incubation period.

Loeffler and Frosch were taken aback, because this was not at all what they had expected. They made repeated control experiments, and the findings were confirmed again and again. To Loeffler and Frosch there were only two possible explanations: either the lymph contained an extraordinarily potent toxin, or the infectious agent

*Beijerinck uses the expression "sich ernährendes Molekül" which translates as "molecules which feed themselves". However, the context suggests that what he had in mind were molecules with an active metabolism.

was small enough to pass through the pores of what was considered to be a bacteria-proof filter. On the basis of meticulous calculations of the dilutions involved, they came to the conclusion that no toxin could be sufficiently strong to have caused their results. This conclusion was reinforced by the results of work carried out almost simultaneously in the laboratories of the Institut Pasteur in Paris.[6] The disease under investigation here was bovine pleuro-pneumonia, and its agent is known now to be a mycoplasma,[7] not a virus. The fact that it was at this early stage recognized as a "filterable agent" probably to some extent delayed further development of the virus concept in the first decades of the century. That it was possible to cultivate the causal organism, albeit under rather special conditions, and to see it, although only just, under the microscope, gave encouragement to those who believed, as did many veterinary and medical bacteriologists working at that time on "filterable viruses", that the failure to observe viruses with the aid of the microscope and to grow them *in vitro* represented only temporary difficulties, to be resolved as techniques improved.

By the turn of the century the concept of the virus as a separate biological entity had emerged from its background of general bacteriology, but still only in a tentative way. Four infectious agents had been shown to pass through filter candles; in addition to the agent of the mosaic disease of the tobacco plant and those of the two cattle diseases of foot-and-mouth and bovine pleuro-pneumonia they included the pathogen of rabbit myxomatosis, a disease first observed and described by Sanarelli[8] when it attacked his stock of experimental animals in Montevideo. The main criterion was filterability; "invisibility in the light microscope" was already questioned by the inclusion of the pleuro-pneumonia agent; and except for Beijerinck, nobody seemed inclined to regard the failure to grow on artificial media as a criterion of necessarily lasting importance.

In the year 1900 a fifth pathogen joined the group. It was the virus of African horse-sickness, described and shown to be filterable by M'Fadyean[9] of the Royal Veterinary College, London. The samples of infected blood had been brought to M'Fadyean from South Africa by an English veterinarian, having been drawn aseptically and preserved with a mixture of glycerol and water in sealed bottles. M'Fadyean's success in demonstrating the continued infectivity of the blood after filtration (filters Berkefeld and Chamberland F) illustrated another of the characteristics of the viruses as opposed to the bacteria, viz., their degree of resistance. The paper does not state the actual duration of the voyage, but in the year 1900 it must have been considerable. M'Fadyean did not initially speculate on the nature of the new virus; he refers to it as a "microbe", and considers that it "does not attach itself to the red corpuscles of the blood". However, in a later review article[10] he discussed the possible nature of the filterable viruses in general. In common with most veterinary and medical workers in the field at the time, he does not seem to have seriously considered any concept other than the microbial one. Although he found the failure of most of the known viruses to grow under artificial conditions difficult to reconcile with an essentially bacterial nature ("one does not see why the ability to grow under artificial conditions should in any degree be dependent on its size"), the suggestion that they might belong to the protozoa (because of their higher degree of resistance to certain chemicals and disinfectants[11]) seemed to M'Fadyean

only to add to the difficulties of the concept as a whole. As for Beijerinck's hypotheses, M'Fadyean writes in his paragraph on the tobacco mosaic virus "The question whether such [Beijerinck's] inferences were justified by the result of the experiment will afterwards be discussed . . .".[10] In fact, M'Fadyean does not refer to Beijerinck's ideas in his later discussion in the same paper. Thus by 1900, with five "viruses" proved "filterable", Beijerinck had gained no support for his views from other workers in the field.

Then, in 1901, three papers were published in rapid succession, all reporting successful filtration experiments with the agent of fowl plague.* This threat to stocks of domestic fowl had only recently come to be recognized as being aetiologically different from chicken cholera, for which the causal organism had been isolated by Perroncito in 1878[12] and attenuated by Pasteur shortly afterwards.[13] Two of the papers were by Italians, published as reports to the academies of medicine in Ferrara, by Centanni and Savonuzzi,[14] and Modena, by Maggiora and Valenti;[15] they became more generally known when German versions[16,17] appeared within the subsequent two years. The discussion below includes additional material from the German versions. The third paper was by the Austrians Lode and Gruber.[18] It describes an outbreak of fowl plague which occurred a couple of months after the Italian ones, and the authors indict "a certain Salvatori, bird-seller of Italy", who was alleged to have brought the fowl plague across the Alps with his stock, and hence to have caused a violent epizootic among fowl in the upper Inn valley.

The almost simultaneous appearance of these three different and very detailed papers on fowl plague virus reflects not only the rapidly growing interest in general in the nature and aetiology of the filterable viruses; more specifically it heralds the importance fowl plague was to assume in years to come as a representative model virus for a number of reasons. Centanni pointed out in 1902 its superiority with regard to the convenience of the experimental work involved; both concerning cost and availability, chickens had obvious advantages over cattle and horses as experimental animals, and the infected fowl plague blood was a convenient medium compared to the brain tissue suspensions which were proving cumbersome in rabies studies.

Apart from the clinical and histological detail, of which there is a great deal, the focal points of all three studies remained the "invisibility" and the "filterability" of the agent of fowl plague, demonstrated in all three cases after conventional bacteriological methods had failed to produce results. In view of the continued virulence of highly diluted infected blood, and of blood after several passages, Maggiora and Valenti[17] concluded that the agent behaved as a "true virus" which multiplied in the infected host, and not as a mere toxic substance.

These authors do not otherwise commit themselves on the subject of the nature of the virus involved; but for its interest in the light of later development, the following passage should be singled out. Maggiora and Valenti wrote "Diseases occur among

*The virus of fowl plague (German: *Klassische Vogelpest*) belongs with the influenza A viruses to the group of myxoviruses distinguished by a stage of synthesis in the cell nucleus and should not be confused with the virus of fowl pest (German: *Atypische Pest*) or Newcastle disease which like the mumps and parainfluenza viruses multiplies only in the cytoplasm.

the hens at the same time as outbreaks of pneumo-enteritis among swine and of other septicaemic forms among various domestic mammals, and fashionable pet birds may in certain circumstances dangerously transmit a serious and often fatal infection to humans".[17] More than fifty years later the virus of fowl plague was shown to be an avian variety of the influenza A virus,[19] and recently recombination of genetic characteristics derived from two different types of influenza virus, one of human and the other of avian or mammalian (other than human) origin, has been suggested as a possible cause of the continual occurrence of novel strains of influenza.[20] The so-called "hog-flu" was first observed among swine in the American mid-west in the autumn of 1918, and may well have been an off-shoot of the great human pandemic.[21] The statement by Maggiora and Valenti is admittedly vague, but the fact that it is coupled with a reference to what must be psittacosis, first described from an outbreak in Switzerland in 1880[22] and subsequently in Germany, Switzerland, Italy and France, does suggest that they considered the spread and interaction of infections between species.

The paper by Lode and Gruber[18] is shorter than either of its companions, but the authors do consider in detail three distinct possibilities for the nature of the virus, and discuss their attitudes to all three. Like Loeffler and Frosch with regard to the foot-and-mouth disease virus, they reject the idea of a highly active, soluble bacterial toxin because of the positive results of serial transmission. On the other hand, the alternative offered by Loeffler and Frosch, i.e. that the agent is a living organism too small to be retained by the filter, is not immediately acceptable to Lode and Gruber. They make a third suggestion which is conceptually closer to the ideas expressed by Beijerinck; but instead of Beijerinck's bold foray into thinking in molecular terms, they invoke humoral as opposed to cellular pathology and propose that "the virus in these perplexing infections may be not corpuscular at all, but rather a dissolved substance able to multiply, something like an enzyme acting through the processes of decomposition which it initiates in its host without being itself consumed". The authors add that the concept of enzymatic bodies passing through filters presents no difficulties for them, since they can relate to the similar cases of toxins and albumens; but as for the final concept of a dissolved body endowed with the ability to reproduce itself, they are concerned about the lack of analogy and basis for comparison.

Less than a year later Lode[23] had carried out a series of comparative filtration experiments; he found that while the fowl plague virus passed without difficulty through the pores of Berkefeld filters, which are made of diatomaceous earth, the results obtained with porcelain filters of different pore sizes* showed that the ease of passage varied with the pore diameter. Lode, who had believed the retaining of micro-organisms by filters to depend largely on the degree of adsorption on to the filter surfaces, was surprised to find that in fact the diatomaceous earth filter with its more extensive internal surface area let the virus through more easily than did the porcelain filters. To Lode this was conclusive evidence that he was not dealing with a dissolved substance, and he decided in favour of the "ultrasmall bacteria" concept of Loeffler and Frosch.

*Different grades of Chamberland filters, as well as Pukall, Hauser and Kitasato filters.

After explaining his preference for the term "filterable" as opposed to "ultravisible" as a means of characterizing the new type of agent, Centanni in 1902[16] discussed the five disease agents so far proved filterable. He had worked earlier on rabies and evidently felt that its virus should belong to the "filterable" class. He tentatively blamed size and adsorption phenomena. Remlinger finally succeeded in passing the rabies virus through a Berkefeld V filter from a suitably dilute suspension of infected brain tissue in 1903.[24]

Centanni's comments are brief and objective; with regard to Beijerinck and the tobacco mosaic virus he merely records Beijerinck's assumption that the virus is in a "liquid" form, but makes no reference to the molecular theory or the attempt to explain an obligate intracellular mode of replication. That this was only because he was unwilling at this stage to commit himself definitively when he considered the evidence insufficient to allow the drawing of conclusions was made clear in his reasoned summing up: "In view of the incomplete methods currently at our disposal, we must of course defer the question of whether the reproduction of this virus involves living organisms or complex chemical molecules, or even elements belonging in some transitional area between the two". This statement is interesting for two reasons. First, it illustrates Centanni's objectiveness and cautious willingness to embrace a wider concept. Second, it reflects the enormous difficulties involved in correlating the accumulated evidence even after much later radical improvements in technique, difficulties which meant that similar statements were still being made fifty years later, so that Bawden was compelled to write in 1964[25] of the oddness of "eminent virologists" continuing to debate as late as 1952 whether viruses were organisms or molecules "years after it should have been obvious that they did not fit easily into either category".

According to Doerr[26] and Bisceglie[27] Centanni did later decide in favour of a chemical concept of the virus entity, and together with his compatriot Sanfelice expounded in Italy the theory of the virus as a type of autocatalytic agent, capable of inducing in the host cell pathologic activities resulting in multiplication of the virus. Sanfelice,[28] using extraction methods recently made available by the work of the physiological chemists,[29] and working with the virus of fowl-pox, found that it had characteristics in common with the nucleoproteids.

But there is yet another aspect of this early work of Centanni's which should be mentioned in a historical context. Centanni made a variety of elaborate attempts to cultivate the fowl plague virus *in vitro* in a specially constructed U-shaped glass vessel. Among the substrates he used were chicken bouillon, filtered extract of hen tissues in physiological NaCl, a mixture of bouillon and chicken serum, whole fowl blood, fresh white of egg and fresh yolk of egg. All were unsuccessful. It then occurred to him to test the infectivity of eggs laid by sick hens, or even taken from hens which had died of fowl plague. Administered orally, mixed with its regular feed over a period of six days, they had no effect on a healthy hen.

Centanni then decided to inoculate embryonated eggs with infected blood in order to observe the effect on the developing embryo. He used a total of four eggs, resealing them after inoculation with paraffin wax and collodion.[30] After twenty-two days of incubation the eggs were opened and examined. One was badly decomposed, another

showed no trace of either an embryo or of any deterioration. The remaining two eggs each contained an imperfect embryo, one of them with a manifest deformity in one eye. A hen subsequently fed on the three eggs which had not been decomposed died of typical fowl plague, though after a prolonged period of incubation. Centanni concluded that the embryo was able to develop in infected eggs but without reaching maturity, and that the virus survived but did not multiply. If he had used a greater number of eggs and pursued this line of thought further and quantitatively, he would have anticipated some later important developments in virus research such as the culture of viruses on the chorioallantois of the developing chick[31] and the method of titrating fowl plague virus by measuring the rate at which it kills developing embryos.[32] Possibly inadequate incubation methods and contamination problems were discouraging further work at the time. In any case, Centanni did not go beyond the four eggs experiment, which nevertheless remains the first recorded instance of inoculation of the embryonated egg with fowl plague virus, and of the use of eggs in virus research in general.

During the next ten years, every attempt to isolate and study an unknown pathogen included an attempt at filtration. Review articles began to appear, recording the rapidly growing number of known "filterable agents". It is a measure of the activity in this field that when Roux[33] published the first of these articles in 1903 he included a total of nine known viruses. When his colleague Remlinger reviewed the situation three years later[34] the number had doubled. The very titles of the essays reflect the uncertainty with regard to classification and characterization which beset virus research and which was to prevail for many years to come. Roux's article is entitled "On the so-called 'invisible' microbes"; Remlinger's is "The filterable microbes"; and M'Fadyean, five years later, preferred "The ultravisible viruses".[10] "Filterable viruses" was to persist for a very long time as the accepted generic term, and was still the title of the classic textbook edited by T. M. Rivers which was first published in 1928 and which included the agents of a variety of disparate infectious diseases.

Nevertheless, since Beijerinck first proposed the obligate intracellular mode of replication for the tobacco mosaic agent, there was a slowly growing awareness that this criterion might possibly apply to other members of the group, and that it might even finally prove to be the crucial one. On the other hand, many attempts were being made to grow various viruses on artificial media in the Loeffler and Frosch tradition. Centanni had failed to grow the fowl plague virus in whole blood; Marchoux[35] reported in 1908 his success with what he considered to be *in vitro* culture of the same virus. His method consisted in depositing 10 cc. of defibrinated fowl blood on a peptone-glucose gel in a test tube, and then inoculating the mixture with 1 cc. of virulent fowl plague blood. When he found the virus to multiply under these circumstances, he concluded that a zone was established in which chemical exchanges between the defibrinated blood and the gel provided the virus with the required quantities of sugar and peptone and chemical constituents from the blood, and that consequently it was growing on an artificial medium. However, it was soon pointed out by Landsteiner and Berliner[36] that the necessary factor was probably the presence of whole intact blood cells, and not just some chemical substance released from them. Although a growing number of microbiologists suspected that the viruses might be

obligate intracellular parasites, it is not at all clear when it came to be recognized as a fact. As late as 1933 Burnet and Andrewes wrote:[37] "The history of bacteriology and the existence of the micro-organisms of pleuropneumonia and Agalactia which have similarities with viruses and yet can be grown on artificial media suggest the possibility that additional information from the areas of physiology and cell metabolism might make it possible to compose artificial media which would accommodate the growth and reproduction of at least some viruses". Hence, in the end, the problem of whether viruses are able to multiply outside the living cell becomes one with that of definition, which was not and could not be finally resolved until the momentous advances of electron microscopy, X-ray crystallography and molecular biology after the second world war.

In this connexion it might be argued that the development of the virus concept has run a close parallel to that of the concept of molecular biology. During the decade before the first filtration experiments were performed, Kossel[38,39] had succeeded in isolating most of the nucleic acids from cell nuclei in a series of ingeniously designed experiments. When his student, Ascoli, found uracil in 1900, the number was complete and Kossel had on hand all the nucleotide bases from DNA and RNA; only the carbohydrate component remained unknown until it was identified by Levene thirty years later.[40]

The concept of large molecules presented far more difficulties. Because it could be dissolved and crystallized, and because it contained iron as a distinctive element, haemoglobin was a favourite subject in much early work. Hüfner[41] determined the molecular weight of a one-Fe unit by elementary analysis; the resulting value of 16,669 is reasonably close to the correct one of 17,000 which, for the total of four haem sub-units, yields the molecular weight of 68,000 finally arrived at by equilibrium sedimentation by Svedberg and Fåhraeus.[42] On the basis of such figures, various empirical formulae were proposed and used for the calculation of an approximate value for the diameter of the haemoglobin molecule. The value given in a contemporary textbook[43] and accepted in most of the early attempts to determine the size of viruses by comparative filtration experiments was 2.3–2.5μμ. But the subject was clouded by uncertainties which could not be resolved with the methods then available, and many eminent chemists flatly refused to believe in molecular weights above 5,000, arguing that the existence of crystals was no guarantee of homogeneity and that only chemical synthesis under controlled conditions could yield unequivocal information.[44] As late as 1924, when Staudinger defined his novel concept of the macromolecule, he met with violent opposition, and the concept became accepted only gradually during the nineteen thirties.

From the early days of the filtration experiments, the size of the "invisible" infectious units also exercised the minds of those who were interested in applying the instruments of mathematics and physics to biological subjects, and to determine the lower limits of size for living organisms. McKendrick,[45] in a presidential address to the British Association in 1901, concerned himself with the general implication of a molecular concept of living tissues, concluding that the smallest organisms visible in the microscope "may contain as many as 1,250 molecules of such a substance as a proteid". McKendrick's calculations were based on arbitrary values such as "Take

the average diameter of a molecule as the millionth of a millimetre . . .", combined with the more factual values representing the limits of resolution of contemporary microscopes. Two years later, Errera,[46] also influenced by the discovery of the filterable agents, published speculations along the same lines. Errera had for a long time been interested in the application of the laws of physics and mathematics in cytological studies, and had established "Errera's law", i.e. the principle of the cell wall, at the moment of formation, having the qualities of a liquid film assuming the shape encompassing the smallest possible area.[47]

McKendrick was a physiologist whereas Errera was a botanist, and it was natural for him to ask the question: "Is it conceivable that living organisms could exist which were smaller than bacteria by the same factor as that by which bacteria are smaller than human beings, not to mention such magnificent species from the plant kingdom as the Giant Sequoias of California or certain Australian Eucalyptus trees?" Errera, on the basis of calculations along much the same lines as those of McKendrick, concluded that it is not. Given the discontinuity of an organized cellular structure, granted that living matter consists of small, but not infinitesimally small, molecules, there is a lower limit below which living organisms, even the most undifferentiated ones, which are nevertheless aggregates of complex molecules, cannot exist. On the basis of comparative calculations Errera concluded that even organisms a few hundred times smaller than the smallest known bacteria are inconceivable, and that in all probability even the "invisible microbes" are not very much smaller than the smallest known bacteria. In a postscript Errera acknowledges McKendrick's essay which has only recently come to his notice. He observes that their calculations agree tolerably well and gracefully suggests that although disagreements exist, it would be futile to pursue them "as we are in the realm of hypotheses and approximations". It was a realm in which virology and molecular biology alike were to stay for many years to come.

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