

AN ANALYSIS OF THE HISTORY OF  
INFECTIOUS NUCLEIC ACIDS

by

Mark Weidenbaum,  
University of Connecticut

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## ABSTRACT

This review traces the significant developments in virus chemistry from Beijerinck's (1898) recognition of viruses as distinct bodies to the demonstration of their infectious nucleic acid nature by Gierer and Schramm (1956). The study demonstrates how cytochemical staining methods applied to inclusion bodies served as a useful method for investigating the early years of virus chemistry. The investigation also analyzes the effects of Stanley's misinterpretation of proteins as infectious agents and his dismissal of nucleic acids as being important in the viral infection process. The study reveals that Sanfelice's (1918) observations were lost from central thought but anticipated many later developments. The review includes a discussion of graphical citation indexing as well as a graphical citation index surveying the history of infectious nucleic acids from 1873 to 1960.

## PART I - HISTORY

### Preliminary Ideas

In 1892 D. Iwanowski<sup>44</sup> recognized that the juice of Turkish Tobacco plants having the tobacco mosaic disease remained active after being passed through a Chamberland filter (a standard microorganism filter). Via this work, Iwanowski had demonstrated the existence of what is now known to be a virus. Yet, he chose to regard the infectious agent as bacterial in nature.

Six years later M. Beijerinck<sup>4</sup> repeated Iwanowski's work but interpreted the results in terms of a "contagious living fluid." Therefore, he was the first to recognize the fundamental difference between the "filter passing agent"<sup>4</sup> and ordinary bacteria.

After Beijerinck's work several diseases were determined to be caused by "filter passing agents," which had been termed viruses. In 1898 Loeffler and Frosch<sup>49</sup> first discovered an animal virus,<sup>62</sup> that causing hoof-and-mouth disease in cattle. In 1911 Walter Reed<sup>62</sup> determined that yellow fever was also a viral disease.

The identification of another major family of viruses, namely those affecting bacteria, followed when Twort<sup>82</sup> and d'Herelle<sup>40</sup> recognized the "bacteriophages."

Although viruses were thus recognized as organisms distinct in themselves, their structure and mechanism remained unknown at this time. As W. Stanley<sup>76</sup> stated, "...the general nature of the viruses was unknown and they had been regarded variously as invisible forms of ordinary bacteria, as a new kind of invisible living organism, as protozoa, as unusual products of cellular metabolism, as enzymes, and as different kinds of inanimate chemical substances."

In general, the problems involved in isolating pure virus samples and obtaining conclusive data prevented significant progress. As the reviews by Roux<sup>63</sup>, Wolback<sup>88</sup>, Twort<sup>83</sup>, and Bayon<sup>3</sup> demonstrate no particular theory predominated.

#### Quantitative Studies

Until 1935 the ideas concerning the nature of viruses were largely based on conjecture. However, in that year W. Stanley<sup>74</sup> successfully crystallized Tobacco Mosaic Virus (TMV) protein via ammonium sulfate precipitation. He then confirmed that this virus multiplied only within the cells of certain definite hosts, thereby differentiating from normal pathogenic bacteria. By thus isolating a crystalline "protein" having TMV activity, Stanley facilitated direct measurements of infectivity by correlating the amounts of TMV protein present with the degree of virus activity.

After measuring chemical and physical constants of the crystallized "protein," Stanley concluded that "...the high molecular weight proteins carrying virus activity are characteristic of virus-diseased activity."<sup>74</sup> Hence, his evidence pointed towards the existence of "infectious proteins."<sup>74</sup>

Although Stanley had assumed that his TMV protein preparations were sufficiently pure, Bawden and Pirie<sup>2</sup> showed that liquid crystals of TMV contained 0.5% phosphorus and 2.5% carbohydrates. They noted that these materials were nucleic acids of the "ribose type."<sup>2</sup> They also postulated that the TMV proteins consisted of long fiber-like particles that aggregated to form longer threads.

Despite this work, Stanley maintained that his preparations were good enough and that the phosphorous "impurities" to which Baden and Pirie referred were of little significance. He based this conclusion

on his earlier findings that phosphorus was unnecessary for virus activity. Thus, he maintained that different viruses led to the synthesis of different proteins and that nucleic acids were of minor importance.

By 1942 Cohen and Stanley<sup>17</sup> had determined that RNA particles had an average molecular weight of 300,000 and were highly assymetrical. More importantly, this time Stanley also recognized that Bawden and Pirie's "nucleic acids" were far more important than he had earlier suspected. Cohen and Stanley concluded from their data that viral nucleic acids existed as threadlike molecules, the length of which corresponded to that of the intact virus molecule.

During the mid-1940's emphasis shifted away from proteins and towards nucleic acids as the possible infectious elements of viral infection. Avery then contributed his finding that in *Pneumococcus* the substance which induced transformation of one bacterial type to another "appeared to be...sodium deoxyribonucleate, inducing the synthesis of non-nitrogenous polysaccharide composed of glucose-glucuronic acid linked in glycosidic union."<sup>1</sup>

Although Avery was working with bacteria and not viruses, his discovery of DNA as the "Transforming Principle" stimulated interest in nucleic acids as vectors of infectivity. He had verified that the inducing substance (DNA) and the substances it induced (high molecular weight proteins) were chemically distinct and biologically specific. These findings heightened the possibility that nucleic acids themselves were responsible for infectivity.

In 1952 Hershey and Chase<sup>41</sup> employed radioactive sulfur and phosphorus tracers to prove that the viral protein shell attaches to the cell

wall of its target but does not enter the cell itself. Instead, it injects its nucleic acid core into the cell. Despite these findings Hershey and Chase could not explain how the virus replicated once inside the infected cell.

In an attempt to explain nucleic acid structure, Pauling and Corey<sup>57</sup> postulated that the nucleic acids might form an alpha-helix. Although this prediction was wrong, the idea of a helix contributed to Watson and Crick's<sup>86</sup> demonstration in 1953 that DNA was composed of a double helix held together by hydrogen bonding and van der Waals forces.

Later that year Watson and Crick<sup>87</sup> proposed that the double helix contained a pair of complementary template strands which could pull apart. Each strand could then form a new complementary chain. They suspected that the sequence of bases attached to the sugar-phosphate backbone making up the strands was the code that carried genetic information.

The Watson and Crick model resulted in the interpretation that viral nucleic acids carried their own replication instructions. Once inside their host, they could replicate and take control of the cell machinery for protein synthesis. The viral nucleic acids could then use this machinery for the synthesis of the high molecular weight proteins necessary for their protective shells. Thus, the viral nucleic acid could itself be the infectious agent responsible for disrupting cell metabolism.

#### Proof of Infectious Nucleic Acids

The final proof that nucleic acids were themselves capable of infection came in 1956 when Gierer and Schramm<sup>32</sup> showed that bare

RNA, purified from TMV, was itself capable of inducing infection in the tobacco plant. They stated: "We are thus led to conclude that the infectivity is due to the nucleic acid itself."<sup>32</sup>

Gierer and Schramm's work stimulated subsequent investigation for infectious nucleic acids in animal and bacterial viruses. In 1957 Spizizen<sup>73</sup> attempted to establish that T2 bacteriophage DNA could infect E. coli bacteria protoplasts, but his preparations were impure and his results inconclusive. Later that year Fraser et al.<sup>30</sup> performed the same experiment and presented more evidence that naked bacteriophage DNA could induce infection. However, it remained for Guthrie and Sinsheimer to prove conclusively in 1960 that "protoplasts of E. coli can be infected with the DNA of ØX174."<sup>37</sup> (ØX174 is another bacteriophage.)

Concurrent with this work on bacteriophages, Colter et al.<sup>18</sup> demonstrated that infectious nucleic acids existed in animal viruses. He stated: "Ribonucleic acid isolated from Ehrlich ascites tumour cells infected with Mengo encephalitis virus is infectious, and the ribonucleic acid component, rather than residual intact virus particles, is responsible for this activity."<sup>18</sup>

## PART II - ANALYSIS

### Francesco Sanfelice

While studying Epithelioma Contagiosum (a viral skin disease observed mainly in birds) in 1914, Francesco Sanfelice<sup>65</sup> noted that "it is most interesting to see how a disease can be produced with the nucleoproteide which was extracted from the diseased tissue." In 1928 Bronfenbrenner<sup>11</sup> that Sanfelice had extracted a substance which per-

petuated disease as if it 'were a living virus.'<sup>11</sup> Unfortunately, the limitations of optical, physical, and chemical techniques prevented Sanfelice from obtaining more than speculative evidence concerning the nature of the Epithelioma Contagiosum virus mechanism.

Nevertheless, Sanfelice was apparently the first to suggest that viral infection was due to something other than attack by the intact virus. Rather, he speculated that the "nucleoproteide", not the complete virus particle, was the infectious element. Thus, he anticipated much of what has since been determined concerning the mechanism of viral attack.

#### Inclusion Bodies

One of the earliest clues concerning the nature of viruses centered on the observation that some diseases induced development of cellular inclusions, referred to as inclusion bodies. In 1881 Rivolta<sup>61</sup> observed such inclusion bodies in the cells of chickens having fowl pox. Bollinger<sup>7</sup> made similar observations while working with fowl diseased with Moluscum Contagiosum. In 1894 Guarnieri<sup>36</sup> discovered typical inclusions in cells having vaccinia (small pox virus).

At first these inclusion bodies were mistaken for protozoa. This mistake gave rise to the term "Chlamydozoa"<sup>59</sup>. During the period of approximately 1910 through 1930 this term was used to describe inclusion bodies.

Although inclusion bodies had been recognized since 1873, their specific connection with virus activity was not demonstrated until Paschen<sup>56</sup> did this in 1917. Subsequently, two fundamental theories arose concerning the relationship of inclusion bodies to viruses. One theory

proposed that the inclusions were products of the reaction of the infected cell to the virus. The other theory, which has since been proven, was that inclusion bodies were virus colonies themselves.

#### Cytochemical Staining of Inclusion Bodies

Once inclusion bodies were associated with viruses, the former were tested cytochemically for a variety of substances, including nucleic acids. Consequently, it has been possible to use cytochemical staining methods as a means of exploring the early years of virus chemistry.

The cytochemical test most useful in studying these years has been the Feulgen Reaction, developed by Feulgen<sup>26</sup> in 1924. It is still the single most definitive test for DNA. It involves hydrolysis of the aldehydes in the nitrogenous bases of DNA which is then followed by rosaniline staining.

Another method for testing nucleic acids (usually RNA) involved differential staining. In 1940 Brachet<sup>10</sup> discussed the use of ribonuclease to cleave "pentosenucleic acid" into soluble mononucleotides as a specific test for RNA. The material to be tested was stained via the Feulgen reaction before and after the action of the ribonuclease in order to make sure that no DNA had contributed to the observed results.

Cowdry<sup>21</sup> was apparently the first to apply the Feulgen test directly to inclusion bodies. In 1928 he stated, "...the Feulgen reaction showed both types of inclusions contain little or no thymonucleic acid."<sup>21</sup> Haagen and Kodama<sup>38</sup> used the Feulgen reaction in 1937, getting positive results (indicating the presence of DNA) for "inclusion bodies" and negative results for "elementary bodies."<sup>38</sup> (There is some confusion today about the precise meaning of this statement as the terms "inclusion

body" and "elementary body" are now considered virtually synonymous.

The first proof that inclusion bodies contained DNA came in 1940 when Smadel and Hoagland<sup>71</sup> used a positive Feulgen reaction to prove the presence of DNA in vaccinia-induced inclusion bodies.

#### A Mistake in Interpretation

Since little was possible before the crystallization of TMV, W. Stanley's work was a major step in turning virology into a disciplined and quantitative science.

However Stanley erred seriously in insisting that viruses were pure protein and in initially dismissing Bawden and Pirie's emphasis on "impurities." Ironically, the very "impurities" to which they referred had, in fact, accounted for the infectious activity which Stanley had measured and mistakenly attributed to viral proteins. Due to his prowess at the time, Stanley's misinterpretation of the possible role of nucleic acids directed virus research in the late thirties and mid-forties toward the study of proteins and away from investigation of nucleic acids.

#### Problems in Communications

1. With regard to Sanfelice, why was his original and provocative work, done in 1914, lost from the focus of the scientific world? Since no ideas during his time could be substantiated, why were his ideas ignored while others flourished? A possible hypothesis is that his use of scientific German was very poor. Thus, his contemporaries probably had so many problems with his ill-constructed sentences that they did not seriously consider the content of his work. Hence, Sanfelice's valuable insights did not flourish.

2. It has often been necessary to use critical reviews in order to determine how well given ideas were accepted in the scientific community. In particular, considerable controversy arose concerning the distribution of credit between Spizizen, Fraser, and Guthrie and Sinsheimer for first proving the existence of infectious phage DNA.

The critical reviews on this topic highlight some differences in approach among investigators. While Ravin<sup>60</sup> and Schramm<sup>66</sup> gave Fraser credit for proving infectious bacteriophage DNA, Kozloff<sup>46</sup> and Colter and Ellem<sup>20</sup> did not agree. While Kozloff and Colter demanded more proof, Ravin and Schramm accepted Fraser's evidence as sufficient proof. It appears that Ravin's and Schramm's interpretations were incorrect since they were based on premature assumptions. Ravin's statement "these findings, albeit preliminary, on the infection of protoplasts by viral DNA raise enormous possibilities for the future..." implies that infectious DNA had already been proven. Actually, the first accepted proof of infectious DNA came with DiMayorca<sup>24</sup> in 1959, a year after Ravin wrote his review.

3. The need for improved communication has clearly been demonstrated in reviewing the literature. An example of this is in the article by Bland and Robinow<sup>6</sup> wherein they state, "So far as we are aware, Haagen<sup>38</sup> (1937) is the only investigator who has applied this reaction (Feulgen reaction) to a virus. He stated that the inclusion bodies of vaccinia gave a positive Feulgen reaction, but that the elementary bodies are negative."<sup>6</sup> Indeed, Bland and Robinow were not aware that Cowdry had applied the Feulgen test to inclusion bodies in 1928.

From the above, it is apparent that as the volume of work grows, new and more effective means of communication are needed. One useful approach which will be described is the Graphical Citation Index.

A. Introduction

The accompanying citation index provides a visual means for tracing developments in virus chemistry from 1873-1960. Continuing analysis of the history of virus chemistry is particularly warranted in view of the close relationship between molecular genetics and the study of infectious nucleic acids. This study centers on the events leading up to the demonstration of the infectious nucleic acid nature of viruses.

Such analysis clarifies and readily exposes historically significant developments by pointing out changes of ideas in addition to new experimental proceedings. Moreover, such interpretation serves as a working tool for re-evaluating early insights and possibly minimizing repetitive experimental work.

The index has several features which are briefly outlined below.

B. Chronological Perspective

A broad overview of the index clearly shows periods of high activity during 1935-1942 and 1953-1960. These time periods, which show much higher "publication density" than the periods 1873-1935 and 1943-1953, generally follow some critical investigation that made available new materials or concepts. For example, Stanley's 1935 TMV crystallization (reference 74) essentially sparked the high "publication density" that ensued from 1935-1942 since it provided a previously unavailable material, the crystallized Tobacco Mosaic virus. Similarly, Hershey and Chase's labelling experiments in 1952 (reference 41) together with Fraenkel-Conrat's work on TMV structure in 1955 (reference 28) stimulated the high "publication density" from 1953-1960 by providing new empirical and theoretical input concerning

the mechanism of viral attack.

Although there may be some omissions, this graphical method physically displays the general periods of activity as well as those of relative passivity.

C. Clustering

The central power of this index lies in that it reveals which authors commonly cited the same references and thereby determines the common-reference-clustering-pattern for each article. Such clustering patterns may then be used to determine the relatedness of different articles according to their degree of common citation. If two articles cite a common reference, they are probably related to each other. Otherwise, they would not have cited the same work. If two or more articles cite two common references, they are almost certain to be closely related. Hence, as their number of common citations increases, the probable relatedness of two (or more) articles also increases.

The determination of relatedness by use of the graphical display is of great use in searching the literature. Using the index one first determines the clustering pattern for a given article. Having then found several articles with at least one reference common to that initially given (and probably many more) one can directly examine these and bypass much of the mass of unrelated material that usually accompanies a literature search.

This method clearly depends on the completeness of the citation index used for the search. The full value of this technique thus increases directly relative to the completeness of the index. Although not exhaustive, the accompanying index provides a starting point for surveying the literature on infectious nucleic acids. It is recommended that this work be more fully expanded.

D. Combination of Techniques

A third service provided by the index is that it highlights where different fields overlap and physically shows where isolated techniques have been combined. New applications of existing techniques has visibly been critical to many of the investigations reviewed here. Some examples include (1) Smadel and Hoagland's demonstration of viral DNA by application of the Feulgen stain to inclusion bodies (reference 71) and (2) Stanley's use of ammonium sulfate precipitation with globulins to precipitate TMV (reference 74).

Thus, by following the techniques cited on the chart, it is possible to decipher where and how different methods came together. This process confirms that important results often follow when two isolated findings are pooled and also brings out the more common instances of lack of communication between investigators.

E. Problems

Presently, there are very few graphical citation indices available. This limits the amount of investigation that can be done with them. Therefore, an important task now is to develop and provide more complete graphical citation systems.

In addition, there are some inherent difficulties which result from the failure by some authors to directly cite original references. Instead, some authors cite secondary references or even none at all if the technique which they involve is very common. This introduces the possibility that the references upon which the index is built may themselves have incomplete bibliographies.

One solution to this problem would be to adopt the convention whereby a formal bibliographic listing would not be necessary to warrant an index connection. Rather, simply mentioning a method

or concept in the body of the paper would suffice for indexing purposes.

Another problem arises from the possibility that authors may cite the same reference for different purposes. As a result, the relatedness of citing articles would not be guaranteed simply by their clustering patterns.

This difficulty mainly affects those articles commonly citing only one reference. As long as one or two additional common citations exist this problem is insignificant.

F. Summary

Despite its problems listed above, the graphical citation index is a powerful tool for analyzing developments in their proper historical framework and for facilitating rapid and accurate literature searches.

This method is universally applicable to all areas of study and provides an immediate picture of how past events have shaped a given field. Hence, it is an excellent teaching tool .

A complete catalogue of citation indices covering specific topics and sub-topics in well defined disciplines could be one of the most useful investigative tools available.

As all branches of investigation become increasingly complex, corresponding problems arise concerning how to maintain the necessary levels of communication. In such light, the citation system outlined above becomes increasingly important since it highlights particular developments, places them in proper perspective, and facilitates rapid information transfer between different sources.

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Note: The following sources are graphically displayed in the accompanying Citation Index. The key is as follows:

- denotes general reference
- denotes review article or text
- △ denotes key (i.e., highly significant) article

Those articles listed in the Bibliography with an \* are not included on the Citation Index.

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