# Advances in VIRUS RESEARCH

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# THE NEUTRALIZATION OF VIRUSES

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## I. Introduction

Οὐ περὶ τοῦ τυχόντος οὖν ἐστὶν ὁ ἀγών,
ἀλλὰ περὶ τοῦ μαίνεσθαι ἢ μή.

### *A. Aim and Scope*

Science advances, it has been said, by rediscovering the same things every twenty-five years. By this token the immunology of viruses might pass for science—the spate of recent reports comes in the fullness of time after the grand survey of 1937, by Burnet, Keogh, and Lush. It only remains to be seen whether all this amounts to more than mere redescription of what was well observed in the mid-thirties. Improved techniques are barren if not matched by some reluctance to dote on observations unconfirmed by theory.

The 1937 review takes full muster of all viruses then known, and of all their immunological reactions; two pages out of its hundred-thirty-eight are under the heading of "quantitative aspects," dampened by a page of qualifications. For all this the work shines with rare insight, and its general conclusions are as true now as they were twenty-five years ago. Yet it would be futile to treat the problem in the same manner today: no one doubts anymore that antibodies *do* act on viruses, and the body of data has grown to embarrassing proportions in the meantime. Rather, one should ask then whether it is beyond the wit of man to derive the laws of these phenomena and thus bring order and consistency into an area of disjunct empirical information. In this direction lie the answers to two of our more urgent present needs. If successful, this approach is bound to yield simpler and reliable practical means of assessing immunity; and it would also define how far immunochemistry can go in exploring the nature of viruses.

The path is straightforward: it starts out from a number of basic principles and leads, through the elimination of theoretically equally likely alternatives, to a comprehensive model of neutralization. The comfort of inductive reasoning is denied to this approach; experimental observations will be used only as test cases, in the sense that a single well-established exception is sufficient to discredit and show up as insufficient a hypothesis. Inevitably, there will be areas where experimental evidence, one way or the other, is still missing. The need for explicit statements here is no less: a review, one should like to believe, lives up to its task not by a safe show of indecision but rather by offering as broad and clear a target as possible for future attack.

### *B. Ways*

The elementary act of immunology is the combination of antibody and antigen. This reaction, like some of the better understood phenomena in physics and chemistry, is beyond the resolution of instrument or method yet devised, and is interpreted on the basis of its side-effects, complications, immediate or long-term consequences. Indeed, a stage least removed from the basic interaction will not of necessity yield the least ambiguous conclusion, and we have become accustomed to accept answers well-padded with more or less plausible intermediary assumptions. In setting out to examine both assumptions and conclusions, one does well to look for a system where a single combination of antigen and antibody may exhibit most, or preferably all, of the varied modes of interaction. From this standpoint the viruses are perhaps the class preferred to all—there is hardly a reaction known to immunologists not demonstrated at one time or other on some virus and its antibody.

Not all reactions belong to the group which will be defined as neutralization, but all are of interest to the extent they can tell us something about the mechanisms shared in common.

*1. Direct Binary Reactions*

Interactions that do not go beyond the combination of the antigenic area proper and the complementary region of the antibody molecule will be referred to as direct binary reactions. This is the only way simple haptens and the corresponding antibodies can interact, and this makes them the classical, though least natural, objects of immunology. In such systems one of the free reactants can be separated by equilibrium dialysis, ultrafiltration, selective precipitation, or in appropriate electric and gravitational fields. Alternatively, the changes in over-all concentration may be followed by measurement of light scattering, osmotic pressure, etc. There are also means of attaching one or the other reactant to inert bulky carriers such as cellulose derivatives, red cell ghosts, or colloid particles, thus allowing ready separation of the uncombined reactant. Without stretching the definition of binary reactions too far, the fixation of complement and allied phenomena may be included in this class. The basic reaction here is the same, and the use of complement and a hemolytic indicator system amounts essentially to a specific color test for the formed antigen-antibody complex.

*2. Sequential Binary Reactions*

Most serological reactions, however, do not stop at the primary stage. It is the exceptional antigen that can be shown to be univalent, and the evidence for bivalent antibodies is by now overwhelming. In this situation it is only to be expected that in the course of its thermal movement the binary complex of a single antigenic particle and a single antibody molecule will meet free antigen and free antibody, and that some of the collisions will be fruitful. By way of such sequential binary reactions large aggregates may be formed, leading to visible precipitation or agglutination of antigenic particles. The technical convenience of such systems has been extended by a variety of methods, and the extent of submicroscopic bridging of two antigenic areas by an antibody molecule can be judged by microscopic observation or even the naked eye if the antigen has been first attached to, say, red blood cells, fine glass beads, or minute spheres of some suitable synthetic polymer.

Notwithstanding their proven usefulness in serology and reliability in immunochemistry, the intimate mechanism of these reactions is far from clear. By the nature of things they occur only at high and approximately equivalent concentrations of the reagents. This bars recourse to the

usual approximations whereby almost any mechanism can be treated, with impunity, as pseudo-monomolecular. Furthermore, the steric conditions within these aggregates are such that, on the one hand, the common assumption of randomness is no longer tenable and, on the other, complex and not readily assessed interactions become possible.

*3. Ternary Reactions*

In addition to immediate effects of antigen-antibody unions, the failure of an antigenic entity to realize any one of its physical or biological potentialities may serve as criterion of immunological interaction. Reactions of this class differ from the previous two in kind, not necessarily in complexity. Besides the basic reactants we have here also a third component, competing with antibody. Such ternary systems may be observed in as many ways as there are distinguishable characteristics of an antigen. Historically, the term *neutralization* covers only the abolishment of toxic action or of infectivity, usually in connection with microorganisms. This restriction does not seem useful, either on logical or on pragmatic grounds. The inhibition by antibody of any enzyme whatsoever from acting on its substrate does not differ in principle from the neutralization of, say, a hemolytic venom. Neither does antibody necessarily act through different mechanisms when inhibiting the agglutination of erythrocytes by a plant extract, or when neutralizing infectivity by preventing the adsorption of some virus to a susceptible cell.

A ternary system has, of needs, more variables than are associated with binary reactions. Also, largely due to the nature of the indicator systems, the measurements are usually less accurate and often more cumbersome. For all that, many effects are brought about by such minute amounts of the antigen that in sensitivity neutralization tests exceed, often by several orders of magnitude, most reactions practicable in physics or chemistry. More to the point, at concentrations of one reagent as low as these, the concentration of the others may be legitimately taken as constant over the whole reaction. Thus an inherently complex system may turn out in practice easier to interpret than many a simpler one working at high and shifting concentrations of its components. Moreover, interactions between molecules, as well as sundry statistical complications due to crowding, are all but absent at this level, and the usual assumptions of ideal behavior are therefore less likely to be contradicted.

## *C. Means*

The process of neutralization could be demonstrated on many viruses, and certain of its aspects would perhaps appear less obscure in some

systems than in others. But choosing didactically convenient examples only condones what Szent-Györgyi deplored as the *clair-obscure* of contemporary science: next to thoroughly worked detail there are patches of indifference and confusion. Arguments built on isolated cases must always range over such immunological no man's land; and no matter how brilliant the documentation, this would be no substitute for coherence. It is well to remember that tests of validity are, on the whole, tests of consistency. Particular propositions within a hypothesis are accepted if they fit in with the rest, that is, if they do not upset the state of mutually unstrained relationships within the system. Obviously, such tests are the stricter and the more informative the denser the net of connections.

*1. Criteria of a Model System*

Since we are to look into the reaction between virus and antibody as revealed by the behavior of the third member of a ternary system, the criterion of coherence imposes certain demands on the material to be used for purposes of demonstration. First, it has to be *homogeneous,* that is, at least one member of the ternary system must remain the same throughout, so that observations can be equated through a common factor, and not compared by analogy. Second, it has to be *extensive,* that is, the best system is the one which has the largest variety of third components by whose response the basic antigen-antibody interaction can be studied. Third, it has to be *intensive,* that is, it must offer the largest number of ways by which the single components and the relationship of any two of them can be defined. This third criterion is necessary since the behavior of a ternary system, complex in itself, can always be analysed into the properties of its three elements, into the pairwise interaction of these, and into the triple interaction. Such conceptual subdivision is more than a matter of formalism or convenience: it is the only way in which tests of consistency can be applied at each level.

The first principle is automatically satisfied by using a single virus as reference material. Common courtesy demands that choice should fall on one of the handful of better characterized groups on which the Commission on Nomenclature has bestowed its approval by way of a generic name in bastard Latin. The second principle calls for a multiplicity of properties which can be inhibited by antibody. In this regard the viruses of influenza stand out: their infectivity for several hosts, toxic action, interfering capacity, enzymic activity, and hemagglutinating property are all readily assayed and neutralizable. No other virus can boast of a comparable combination of properties. By the third principle, once again, influenza is the virus of choice. Its specific relation to a variety of cells

and receptor analogs is well understood, and the nature of interaction can be modified at will by enzymic or chemical treatment of the receptive surface or of the virus itself. There is an almost unlimited series of natural antigenic variants in this group, showing all degrees of serological crossing. Further stable antigenic mutants can be selected in the laboratory, and particles carrying antigenic mosaics have been obtained by genetic recombination. The basic reaction with antibody is open to study by precipitin techniques, gel diffusion, agglutination of virus-coated cells, complement fixation, and, more recently, by direct physical separation of the reactants. This combination of approaches is unique in the virus field.

Thus, for the same reasons that we chose influenza viruses for work on neutralization in our laboratory, these viruses will serve here as the model system on which the mechanism of virus-antibody interaction can be best tested.

*2. The Virus*

The viruses of influenza are small spheres of approximately 80 mμ diameter (Taylor *et al.*, 1943a,b, 1944; Sharp *et al.*, 1944a,b,c; Lauffer and Stanley, 1944; Williams and Wyckoff, 1945; Morgan *et al.*, 1956). Electron microscopy after negative staining (Brenner and Horne, 1959) has recently revealed their characteristic surface pattern (Horne *et al.*, 1960; Hoyle *et al.*, 1961; Choppin *et al.*, 1961) as an array of squat rods projecting through a less well-defined outer membrane. These structures must carry all properties bound up with reactions of the intact particle, such as its external antigen, the areas responsible for specific adsorption to cellular receptors, and the enzymic activity associated with this group of viruses. Although the elementary particle can be broken up by ether treatment (Hoyle, 1952) or more effectively by mild detergents (Laver, 1961a), separation of the antigenic, adsorptive, and enzymic components has not been achieved. This does not imply that all these properties are different facets of the same structure: the physically separable units have molecular weights of the order of a million, whereas end-group analysis (Laver, 1961a) shows that about a quarter of the viral protein is in subunits of circa 35,000 molecular weight, and characterized by aspartic acid as N-terminal. The remainder, with no amino but tyrosine and leucine as C-terminal, still appears to be made up of similar subunits, as evinced by the peptide maps (Laver, 1962) of the fractions. It is thus best to think of the viral surface as a repeating pattern, with each of the various properties multiply represented in the form of identical subunits.

The surface antigen of the virus is strain specific and exists in a

large number of variants. It has been frequently observed that even within a local outbreak several related but distinguishable antigenic types coexist (Magill and Sugg, 1944; Burnet and Stone, 1946), and mutability has been proposed as one of the cardinal traits of influenza (Smith, 1949, 1951, 1952; Burnet, 1950). Major changes have occurred every ten to twenty years, with resultant pandemics. Whether the range of possible antigenic variants is unlimited or goes through secular cycles has been debated over the last quarter of a century; the latter view has on its side the esthetically more satisfying hypothesis, the former the facts.

In contrast to antigenic plasticity, the way virus and cell interact remained notably stable over the years. Starting with the discovery that the virus particle was specifically bound to and capable of agglutinating red blood cells (Hirst, 1941; McClelland and Hare, 1941), the classical studies of Hirst (1942a,b, 1943; Hirst and Pickels, 1942) laid the foundations both for an understanding of the earliest stages of infection, and for a powerful technique, the hemagglutinin test, still the simplest and most accurate of all assays in animal virology.

The same studies led Hirst also to the recognition that the virus particle behaves like an enzyme in its reaction with cells: after combination with some component on the cellular surface (receptor), the virus elutes spontaneously, leaving the cell incapable of binding further doses of virus. The eluted particles are just as active as before in destroying receptors on new lots of cells. Intensive work on this phenomenon by Burnet and his school further revealed that the firmness of combination between virus and cell is variable and characteristic of the particular virus and cell (Burnet *et al.*, 1945, 1946; Burnet, 1945; Ada and Stone, 1950a,b; Stone and Ada, 1950, 1952); that several bacterial species produce a homologous exoenzyme (Burnet *et al.*, 1946; McCrea, 1947; Stone, 1947a; Burnet and Stone, 1947); that infectible cells lose their adsorptive capacity and are rendered insusceptible to infection *pari passu* (Stone, 1947b, 1948a,b; Fazekas de St.Groth, 1948a,b); that there exist soluble receptor analogs, in every respect comparable to cellular receptors (Burnet *et al.*, 1947; Burnet, 1947, 1948a,b,c; Anderson, 1948; McCrea, 1948; Anderson *et al.*, 1948; Gottschalk and Lind, 1949a); that the combination between virus and cell can be made permanent by chemical treatment of the cell (Fazekas de St.Groth, 1949; Fazekas de St.Groth and Graham, 1949) or by using enzymically inactive virus (Burnet, 1952). Eventually Gottschalk and Lind (1949b) isolated the split product, Gottschalk (1951, 1954, 1956) characterized the substrates as mucopolysaccharides carrying terminal sialic acid residues, and demonstrated that the enzyme cleaves the $\alpha$-ketosidic linkage between

N-acyl neuraminic acid and the aglucon, usually galactosamine (Gottschalk, 1957). Hence the enzyme of influenza viruses is a neuraminidase.

*3. Antibodies*

The classical type of antibody is a component of blood plasma, and increases in quantity on specific antigenic stimulation. Antibodies can be precipitated and sedimented as globulins (Marrack and Smith, 1930; Breinl and Haurowitz, 1930; Goodner and Horsfall, 1937; Kabat, 1939), and their electrophoretic behavior classes them with the γ-globulins (Tiselius and Kabat, 1939). This is an inhomogeneous group, and recent work (Kuhns, 1954, 1955; Porter, 1955; Humphrey and Porter, 1956; Askonas *et al.*, 1956; Sober *et al.*, 1956; Porter and Press, 1957) shows that the same serum may contain at least two electrophoretically separable species of antibody molecules, their presence and ratio depending on the history of immunization. From the frictional coefficients and sedimentation constants, the length and width of antibodies is estimated to lie between 200 and 300 A. and 38 to 43 A., respectively. Direct electron microscopic observation of the spacing of virus particles precipitated by antibody (Anderson and Stanley, 1941; Schramm and Friedrich-Freksa, 1941; Malkiel and Stanley, 1947; Hall *et al.*, 1959) confirms these values. More accurate information, both on structure and composition, has come from Porter's work (1955, 1958a,b, 1959). He has shown that antibodies can be broken down into three components. Two of these are similar and carry antibody specificity. The third (central ?) component is larger, crystallizes readily, has no antibody activity, but carries the antigenic determinants of γ-globulins. Respective molecular weights are $50 \pm 1.6$, $54 \pm 2.1$ and $80 \pm 1.6$ thousand, giving an over-all molecular weight of $185{,}400 \pm 1400$ (Charlwood, 1959). The area on the antibody molecule endowed with immunological specificity is, from all evidence, a small region only, spatially corresponding to a surface of about 200 $A^2$., that is, to an antigenic determinant of about 500–1000 in molecular weight (Landsteiner and van der Scheer, 1938; Haurowitz and Schwerin, 1943; Campbell and Bulman, 1952; Kabat, 1954, 1956, 1957; Karush, 1956).

*4. Assays and Indicators*

The interaction of antibody and influenza viruses can be detected directly in the form of precipitates (Magill and Francis, 1938; Henle and Chambers, 1941; Takátsy, 1954; Belyavin, 1955, 1956, 1957). The reaction, highly specific and suitable also for antigenic typing, requires concentrated and preferably purified virus. In its more refined form, allowing bands of precipitate to develop in agar gel, Jensen and Francis (1953) could distinguish several antigen-antibody systems in the one

test, especially when using postvaccination sera. Immunodiffusion has proved particularly useful when looking for impurities, minor antigenic components, or heterogeneity within the antibody population. All these techniques are relatively insensitive: visible precipitates demand high concentrations of both antigen and antibody. Micromethods have been developed by evaluating precipitin tests under the microscope (Smith *et al.*, 1956), and by setting up gel diffusion on a micro scale (Grasset *et al.*, 1956, 1958). With influenza virus all these techniques are liable to inhibition by serum components (Belyavin, 1957), and thus may on occasion give false negative results.

The sensitivity of aggregating techniques can be raised by over a hundredfold if the antigenic particle is firmly attached to some inert carrier. Periodate-treated red cells afford such a system: influenza viruses are bound to these cells but can elute neither spontaneously nor by the action of bacterial neuraminidases (Fazekas de St.Groth, 1949). The method is the artificial analog of the virus-carrying cells naturally obtained with Newcastle disease (Burnet and Anderson, 1946) and mumps viruses (Burnet, 1946), and falls into the general class of passive hemagglutination as practised by adsorption of various antigens to normal (Keogh *et al.*, 1947, 1948) or tanned (Boyden, 1951) red blood cells. No inhibitors of hemagglutination were found in this system, but all normal sera contain low-titer agglutinins for virus-coated cells, setting a baseline of false positive reactions.

The elementary complex of influenza virus and its antibody fixes complement (Smith, 1936; Fairbrother and Hoyle, 1937; Hoyle and Fairbrother, 1937; Lush and Burnet, 1937). Over the years this reaction has proved one of the most reliable tools in assaying and comparing antigenic structure (Henle *et al.*, 1944, 1958; Henle and Wiener, 1944). Micromethods have been devised, both for simple titration employing constant complement (Donnelley, 1950) and, especially, for accurate estimation of antigenic relationships in the form of factorial tests with back-titration of residual complement (Fulton and Dumbell, 1949; Fulton, 1958; Belyavin, 1953). On the whole, complement-fixing techniques are not vitiated by interfering side reactions, and their specificity and sensitivity is beyond question. Why they continue to be bypassed for less reliable techniques must remain a puzzle to the open-minded onlooker.

Historically, ternary assays were developed in the order of decreasing complexity: neutralization of infectivity first, then inhibition of hemagglutination, and finally, the simplest—inhibition of enzyme activity. The indicator systems employed in these tests are of great variety. The virus of epidemic influenza, originally isolated in ferrets (Smith *et al.*,

1933), was soon adapted to mice (Andrewes *et al.*, 1934) and to the chorion of embryonate eggs (Smith, 1935; Burnet, 1935). Neutralization tests for each of these host systems were developed using survival time, number of survivors, number of infected centers, extent of lesions, and development of immunity as test criteria. Thus, even during the first decade of experimental work on the virus, both quantal and quantitative, direct and indirect assays of neutralization were available. The method of Horsfall (1939), based on the combined score of death time and lung lesions after intranasal challenge of mice, was the most accurate and stood the test of time. With the discovery of the amnion (Burnet, 1940a,b) and the allantois (Nigg *et al.*, 1940, 1941; Henle and Chambers, 1941; Burnet, 1941) of chick embryos as highly susceptible hosts, previous techniques were largely superseded, mainly because the virus appeared in quantity in extraembryonic fluids which could be readily sampled and tested for the presence of virus by hemagglutination (Hirst, 1942c,d). These techniques are quantal.

Several lines of stable cells in tissue culture have been tried for susceptibility to influenza. Many were found infectible, in some cases cytoctony marking infection (Henle *et al.*, 1955; Ledinko, 1955), in others the demonstration of hemagglutinin within (Henle *et al.*, 1955) or on the surface (Vogel and Shelokov, 1957; Shelokov *et al.*, 1958) of the cells. None of the systems in this class measures up in sensitivity to the allantois, not to mention the fact that the particles produced in these cells are not released and are noninfective. Primary cultures of embryonic tissues were found to support multiplication of several but not all strains (Mogabgab *et al.*, 1954, 1955, 1956; Granoff, 1955; Haas and Wulff, 1955, 1957; Tyrrell, 1955; Ledinko *et al.*, 1957; Hinz and Syverton, 1959). The most promising of these seems the outgrowth from calf kidneys and fetal pig lungs from which an assay system, either quantal or quantitative, has been developed and proved suitable for immunological studies (Lehmann-Grube, 1962a,b; Lehmann-Grube and Fazekas de St.Groth, 1962).

Large doses of influenza virus kill mice with toxic symptoms within a day or so (Henle and Henle, 1944c), and the abolition of toxicity is another way of observing the effect of antibodies on virus. The phenomenon has been carefully studied (Henle and Henle, 1946a,b, 1948; Mims, 1960), but considerable differences in toxicity were found among strains, and the response also varied from host to host. For these reasons, in spite of its simplicity, the method found little favor in practice.

When virus inactivated by ultraviolet radiation, heating, or treatment with formaldehyde is brought in contact with susceptible cells, a sub-

sequent inoculum of live virus fails to give full yield (Andrewes, 1942; Henle and Henle, 1943, 1944a,b, 1945a,b; Henle *et al.*, 1947a; Ziegler and Horsfall, 1944; Ziegler *et al.*, 1944). The time course (Fazekas de St.Groth *et al.*, 1952; Henle and Paucker, 1958) and quantitative aspects of this reaction have been worked out (Fazekas de St.Groth and Edney, 1952; Powell and Pollard, 1956; Henle and Paucker, 1958). As the extent of interference is measured as yield of hemagglutinin, neutralization of interfering activity (Henle and Henle, 1945b) could, in principle, provide a quantitative test for antibody action in the most susceptible host system, eggs. Since, however, such an assay would involve a two-stage procedure, informative over a narrow range of interfering doses only, the method has not been taken up in practice.

The indicator system in antihemagglutinin tests is the red blood cell. Influenza viruses will agglutinate a wide variety of these (Clark and Nagler, 1943), and the outcome of the test varies considerably with the species of cell used. Some cells not agglutinated under standard conditions become agglutinable on treatment with periodate (Fazekas de St.Groth, 1949), by conducting the test in the cold (Fazekas de St. Groth, 1949; Stone, 1951; Tamm, 1954a,b), or by using heated virus (Briody, 1948). Conversely, the binding strength of readily agglutinable cells can be lowered by graded treatment with neuraminidase, resulting in a notable increase of antihemagglutinin titers (Stone, 1947a). The simplicity and flexibility of this system makes it particularly suited to the study of virus-cell interaction, one of the components of the neutralization process.

Inhibition of enzyme activity affords an equally wide scope for experiments. Taking red cells as substrate, the extent of neuraminidase action can be assessed either by the agglutinability of the cells (Burnet *et al.*, 1946; Stone, 1947a) or by the reduction of their electrophoretic mobility (Hanig, 1948; Ada and Stone, 1950a,b; Stone and Ada, 1950, 1952). Alternative substrates of macromolecular dimensions are available in the family of mucoproteins (serum mucoid: Francis, 1947; Anderson, 1948; McCrea, 1948; ovarian cyst mucin: Burnet, 1947; ovomucin: Gottschalk and Lind, 1949a; Eckert *et al.*, 1949; urinary mucoid: Tamm and Horsfall, 1950; ovine and bovine salivary mucin: McCrea, 1953; Curtain and Pye, 1955). Each of these can be tested by two independent methods, the first relying on their biological activity (Burnet, 1948a,b,c) and the second on the release of free neuraminic acid (Gottschalk, 1951; Warren, 1959; Comb and Roseman, 1960; Warburton, 1962).

Small molecular compounds are also available as substrates (orosomucoid: Weimer *et al.*, 1950; fetuin: Pedersen, 1944, 1947; Klenk and Faillard, 1957; sialyllactose: Kuhn and Brossmer, 1956). Action on this