The origin and development of immunochemistry (1890–1965)

Felix Haurowitz

The concepts of immunity, immunization and immunology were developed as a consequence of Jenner's discovery in 1796 of the protective action of vaccination against blackpox. There was initially no connection between immunology and chemistry.

Almost 100 years later, Emil von Behring and S. Kitisato [1] demonstrated that an immune serum against diphtheria neutralized the diphtheria toxin. This discovery suggested that the reaction between antigen (the toxin) and antibody (the antitoxin) might indeed be a chemical reaction. In 1898 in Frankfurt, Paul Ehrlich advanced his side chain theory of immunity. Later, at the invitation of the Royal Society of London, Ehrlich presented his theory as the Croonian Lecture of 1900 [2]. According to Ehrlich, the parenterally administered antigen (Ag) combines with the homologous antibody molecules (Ab) to form an antigen–antibody complex. Small amounts of the different types of antibodies were assumed to be present in the organism before immunization as preformed antibodies. Ehrlich illustrated his views by the diagram shown in Fig. 1 [2]. The antigenic groups of the cell were designated as haptophoric side chains. The antibody particles were shown as closely adapted to the antigenic side chains. Since they enclosed the haptophoric groups, they were also designated as receptors.

The investigations of Arrhenius

In order to obtain more insight into the nature of antibodies, Ehrlich and the Danish immunologist T. Madsen invited the famous Danish physiochemist Arrhenius to investigate the mechanism of antigen–antibody interaction. Arrhenius [3] published his results in a monograph in which he discussed the equilibria between toxin (T) and antitoxin (A) according to the equation $T + A \rightleftharpoons T.A$. It is evident from this work that Arrhenius considered antigens and antibodies as molecules. Indeed, Arrhenius titled his book *Immunochemistry* and may thus have created this term.

Up to 1930, the only theory of immunity mentioned in textbooks was Ehrlich's side chain theory. It was considered ingenious but highly speculative, as shown by the following quotations from *A System of Bacteriology* published in London in 1931. In volume 6 (Immunology) of this handbook (which, in its time was a highly authoritative text), C. H. Browning stated that the term antibody 'does not correspond with a chemically defined substance' and that Ehrlich's view 'lacks proof'. In the same volume, P. Hartley wrote: 'The chemical nature of antibodies is unknown.' However, the chemical basis of immunological reactions was definitively established by a series of experiments performed at the medical school of the University of Vienna. R. Kraus had discovered in 1897 that proteins act as antigens and induce the production of highly specific antibodies. *In vitro,* these antibodies specifically precipitated the administered antigens. In the year 1906, F. Obermayer and E. P. Pick found that injection of an iodinated protein leads to the production of two types of antibodies; one of these is directed against the iodinated tyrosine residues of iodoproteins; the other is directed against other parts of the protein. The presence of the anti-iodo antibodies was proven by their ability to precipitate iodoproteins that contained other protein carriers. These experiments led to the use of chemically marked antigens in the brilliant work of another Viennese, Karl Landsteiner.

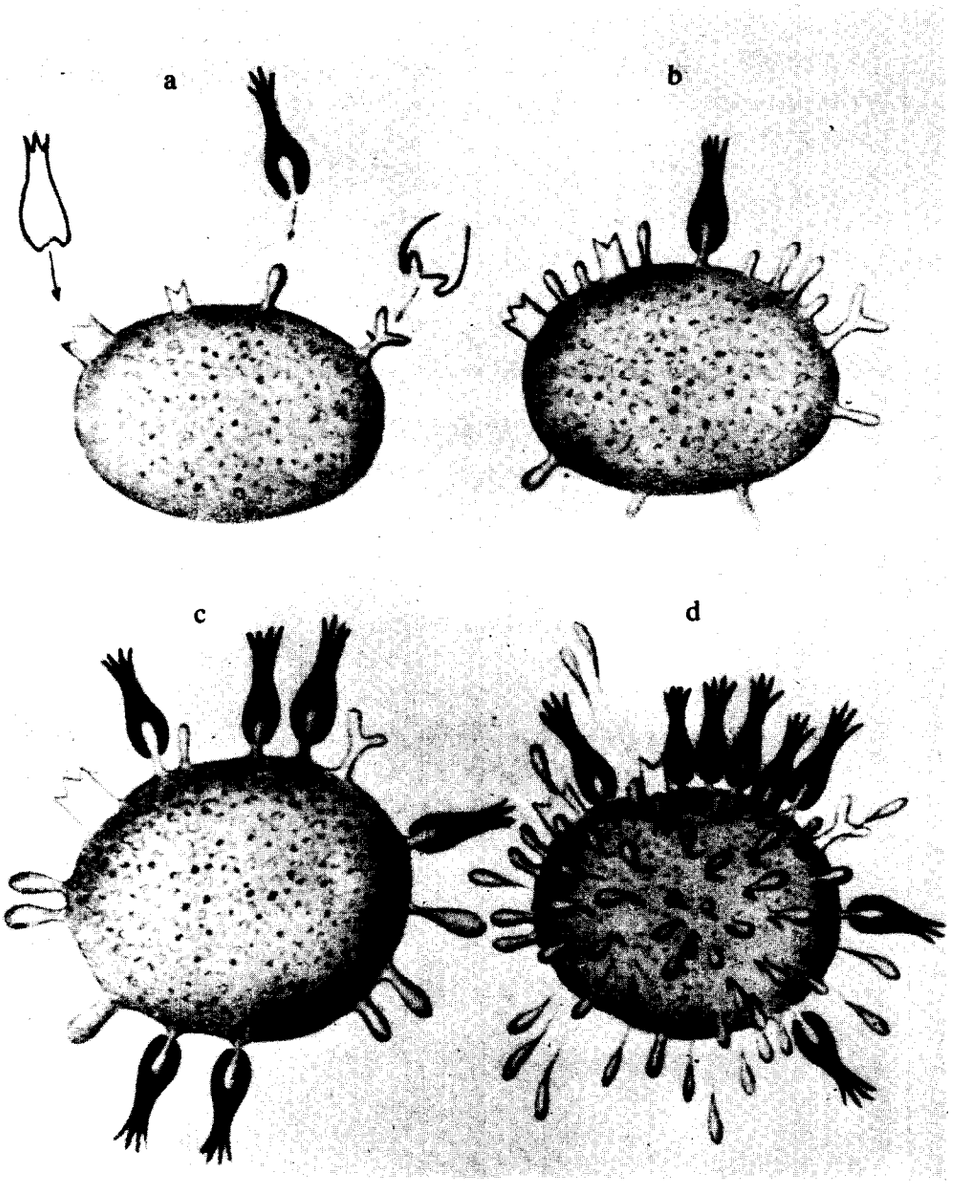

Fig. 1. A lymphoid cell which in the phases a, b, c, *and* d *produce an increasing number of side chains. Some of these side chains are adapted to an antigen (dark particles). If side chains pass into the circulation, they act there as antibodies and combine specifically with the homologous antigens.*

Felix Haurowitz is at Indiana University, Bloomington, Indiana 47401, U.S.A.

Landsteiner and the importance of shape

Landsteiner modified proteins first by alkylation and acylation. He found later that the mildest method of modification was the coupling of proteins with diazonium compounds in aqueous solution. Most of this work was performed after Landsteiner's move from Vienna to the Rockefeller Institute in New York. His work demonstrated that antibodies produced by the injection of optically active *d*-compounds were different from antibodies directed against the stereoisomeric *l*-compounds, and that antibodies directed against *cis*-isomers were different from the analogous *trans*-isomers. Moreover, antibodies against *ortho-*, *meta-*, and *para*-isomers could be differentiated from each other by means of the homologous antigens. It is evident from all these results that the shape and the conformation of an antigen molecule is of great importance for its immunological specificity. In agreement with this view, antigens usually give rise to the formation of antibodies which combine not only with the homologous antigen, but also with antigen molecules having a similar structure and conformation. Most of Landsteiner's work has been described by himself in a classical monograph [4].

In retrospect it is surprising that Landsteiner's fundamental results were based on the very simple precipitin test in which the results were recorded merely by symbols such as +++, ++, +, tr (trace) and – (negative). Quantitative chemical methods of analysis were described in a short paper by Hsien Wu [5]; using hemoglobin or iodinated proteins as antigens, he determined the antigen and antibody content in the precipitate by means of colorimetry. More extensive quantitative data were obtained by Michael Heidelberger and his co-workers [6] who used nitrogen-free carbohydrate antigens; the antibody protein was calculated from the nitrogen content of the precipitate which in turn was determined by the Kjeldahl method, later by colorimetry according to Lowry or by the ultraviolet absorption at approximately 280 nm. In my own analytical work, we used gravimetry with microbalances to determine the total weight of the precipitate; the antigen content was then estimated by means of colorimetric methods [7].

It has been known for many years that the antibody properties are found in the globulin fraction of the immune sera. However, it was not clear whether the specificity of the antibodies was determined by certain groups of amino acids or by prosthetic non-protein groups attached to a protein carrier. In the period before 1940, it was not yet possible to determine the amino acid content of proteins. Only tyrosine, arginine, cystine and a few other amino acids which give typical color reactions could be determined by colorimetry or spectrophotometry. Analyses of this type indicated that the content of these amino acids in rabbit antibodies was approximately the same as in normal rabbit globulins [7]. In 1939, Tiselius and Kabat found that antibodies are γ-globulins and that they occur in the blood serum in the same fraction as the normal γ-globulins. The molecular weights of the two principal fractions of γ-globulins were approximately 1.5×10^5 and 8×10^5. They were later designated as IgG and IgM, respectively; the letters Ig stand for the term 'immunoglobulin'.

In 1936, Parfentjev found that the diphtheria antitoxin can be purified by pepsin digestion at low pH. This digest contains a large active split product in addition to many small inactive peptides. Highly active antibody fragments were obtained in 1950 by Porter [8] when he replaced pepsin by papain and thus avoided denaturation by the low pH required for pepsin digestion. Papain digestion of rabbit IgG yielded a crystalline fragment that lacked antibody activity; it is now called Fc. Most of the antibody activity was found in two fragments originally designated I and II. Later, Nisonoff [9] found that these fragments were artifacts produced from a common precursor by slight differences in the experimental conditions.

The structure of IgG elucidated

The final structure of IgG was elucidated by Edelman [10] who separated subunits from each other by the reduction of interchain disulfide bonds and thus obtained four peptide subunits for each IgG molecule. They are now designated H (heavy) and L (light) chains and are linked to each other according to the structure L–H–H–L in which the horizontal lines indicate disulfide bonds. The presence of four peptide subunits was quite surprising because only two N-terminal α-amino groups had been detected in the IgG molecules. This apparent contradiction was explained by the finding of two N-terminal residues of pyrrolidonecarboxylic acid, the cyclic anhydride of glutamic acid which is free of α-amino groups.

Attempts to sequence antibodies

When the four-chain structure of antibody molecules was clarified, attempts were made to determine their amino acid sequence. These attempts failed because the isolated H and L chains were always mixtures of numerous similar, but not identical, L and H chains. We know at present that this heterogeneity merely reflects the presence of multiple antigenic determinants in the macromolecular antigens. Since one of the prerequisites for immunogenicity is a molecular weight of at least 10^4, antigens need to be macromolecules. If the antigen is a protein, its surface contains many different combinations of amino acid side chains, many of them acting as antigenic determinants. Some almost homogeneous antibody molecules have been found following immunization with those polysaccharides which are polymers of only one or two monosaccharides.

When the protein nature of antibodies had been established, methods of physical chemistry were applied to antibodies in the hope of discovering some particular characteristics of this class of proteins. An excellent survey on physicochemical methods was published in 1938 by Marrack. The best known of these methods was electrophoresis, introduced into immunochemistry by Williams and Grabar [11]. The affinity of antibodies for small homologous haptens was measured by equilibrium dialysis. The sensitivity of these methods was later increased by labeling the reactants (antigens and/or antibodies) with radioactive isotopes. However, none of these methods was able to isolate homogeneous antibody preparations from the heterogeneous mixture of the natural antibody molecules.

The present era of rapid growth and development of immunochemistry began in 1962 with the surprising discovery [12] that the Bence–Jones proteins, which are excreted in the urine of patients suffering from multiple myeloma, are the light chains of the myeloma proteins. The latter are typical immunoglobulins. In contrast to the normal immunoglobulins, the myeloma proteins are homogeneous and thus can be analysed for their amino acid sequences [13,14]. Further analyses of the human myeloma proteins revealed that each patient produces a specific homogeneous myeloma protein, different from the myeloma proteins of other patients. Homogeneous myeloma proteins can also be produced in some animal species. They are easily elicited in mice by the intraperitoneal injection of mineral oil which is a non-specific irritant and not a typical antigen. During the last few years, hundreds of human or murine myeloma proteins have been investigated. Compari-

son of their amino acid sequences has afforded deeper insight into the mechanism of immunoglobulin biosynthesis and into the genetic aspects of immunology.

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The discovery of the superoxide radical

Linus Pauling

Earlier this year Irwin Fridovich and H. Moustafa Hassan (May, 113) wrote of the toxic effects of the superoxide radical. Here Linus Pauling describes how this radical may have been the first important substance whose existence was predicted through arguments based on the theory of quantum mechanics.

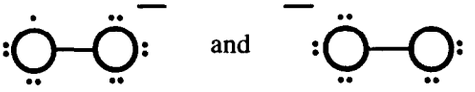
During the last decade I have been mainly interested in the use of vitamin C in preventing and treating viral diseases and cancer. Vitamin C (ascorbate) has the power of inactivating viruses *in vitro*, and Murata and Kitagawa [1] in 1973 pointed out that oxygen is involved in this inactivation, and suggested that the inactivation may occur as a result of the formation of the superoxide radical, O_2^-, or the hydroxide radical, OH. Morgan, Cone and Elgert [2] have reported that superoxide dismutase has no effect on the reaction of superoxide with viruses, but catalase suppresses it. The cleavage of DNA induced by ascorbate is, however, unaffected by superoxide dismutase, although it is suppressed by catalase, so that it is probably the hydroxyl radical that is responsible for scission of DNA strands.

The enzyme superoxide dismutase was discovered only ten years ago [3]. It catalyses the reaction of two superoxide radicals to give oxygen and hydrogen peroxide. Superoxide dismutase is found in all aerobic organisms. Obligate anaerobes, which do not contain superoxide dismutase, are killed in air, and Morgan, Cone and Elgert have suggested that the enzyme played a crucial role in the evolution of life as the partial pressure of atmospheric oxygen increased. The superoxide radical may well be the principal cause of oxygen toxicity, and superoxide dismutase may be essential for survival.

Linus Pauling is associated with the Linus Pauling Institute of Science and Medicine, 2700 Sand Hill Road, California 94025, U.S.A.

During the last five years more papers have appeared on superoxide dismutase than on any other single enzyme (Malcolm and Coggins [4]) and a recent monograph on superoxide and superoxide dismutase [5] contains a history of the enzyme, but no history of its substrate. The superoxide radical may well be the first important substance to have been discovered through quantum mechanical arguments. In the early 1930s I wrote several papers about the nature of the chemical bond, the second of which dealt with the one-electron bond and the three-electron bond [6]. I pointed out that a stable one-electron bond can be formed only when there are two conceivable electronic states of the system with essentially the same energy, the states differing in that for one there is an unpaired electron attached to the second atom. A three-electron bond can be formed between two atoms, A and B, with A having an orbital occupied by a pair of electrons and B having an orbital occupied by a single electron, or the reverse, if the energies of these two structures are essentially the same. The resonance energy corresponding to the interaction of the two structures is the energy of the three-electron bond. It is usually about 60% as great as the energy of a shared-electron-pair bond between two atoms. I carried out a detailed calculation for the helium molecule ion, He_2^+, in which the three-electron bond corresponds to resonance between the two structures He: $\cdot He^+$ and He^{+} :He [7]. At that time, 1931, I assigned to the oxygen molecule in its normal state the structure in which the two atoms are linked together by a single bond and two three-electron bonds.

I had then not yet thought about the possibility of the existence of the ion O_2^-, with a three-electron bond plus a single bond, corresponding to resonance between the two structures:



A few months later this idea occurred to me, and I realized that the ion O_2^-, intermediate between molecular oxygen and hydrogen peroxide, should have enough stability to exist. I knew that when potassium, rubidium and cesium burn in oxygen, higher oxides are formed which were called tetroxides and were assigned the formulas K_2O_4, Rb_2O_4, and Cs_2O in the reference books of inorganic chemistry. I also knew that the tetroxide ion, with the presumable structure of a chain containing three single oxygen–oxygen bonds, would be unstable, because of the well-known instability of the oxygen–oxygen single bond, as represented in hydrogen peroxide [8]. It seemed likely, accordingly, that these substances were in fact KO_2, RbO_2, CsO_2, containing a unipositive alkali ion and the anion O_2^-. A test of this hypothesis could be made by measuring the magnetic susceptibility of the substances, because the tetroxides would be diamagnetic and the compounds MO_2, containing an anion with an odd number of electrons, would have the paramagnetism corresponding to one unpaired electron spin. I accordingly asked a postdoctoral fellow working with me, Edward W. Neuman, to prepare a sample of this oxide of potassium and to measure its magnetic susceptibility. He carried out this measurement, and found the magnetic susceptibility to be that corresponding to