IMMUNOCHEMICAL TECHNIQUES FOR THE IDENTIFICATION AND ESTIMATION OF MACROMOLECULES

2nd Fully Revised Edition

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J. Clausen

The Neurochemical Institute
58 Rådmandsgade
Copenhagen
and
Laboratory for
Biochemistry and
Toxicology
Roskilde University
4000 Roskilde
Denmark

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This book is the revised edition of Volume 1, Part III, of the series 'Laboratory Techniques in Biochemistry and Molecular Biology'.

Volume I of the series contains the following parts:

Part I Electrophoresis of proteins in polyacrylamide and starch gels
by A.H. Gordon – 2nd Edition 1978

Part II An introduction to gel chromatography
by L. Fischer – 2nd Edition 1980 (Gel filtration chromatography)

Part III Immunochemical techniques for the identification and estimation of macromolecules
by J. Clausen – 2nd Edition 1981

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Preface

The aim of this manual is to present practical advice on application of the rapidly expanding mass of data on immunochemistry, and more specifically to examine the possibilities of employing immunological microtechniques in biochemical and histochemical analyses. The terminology in immunochemistry and protein chemistry has been bewildering (Grabar 1963); however, in 1964 WHO and in 1975 The International Union of Immunological Sciences recommended rules on the nomenclature of immunoglobulins (cf. p. 9). The World Health Organization terminology has been used throughout the manual, although this terminology has not, however, been generally accepted in all laboratories working in the field of immunochemistry (WHO 1964).

It is not the object of this manual to present an entire and comprehensive work in the field of immunochemistry, competing with the classical work of Kabat and Mayer (1961), but rather to describe the practical application of immunochemical analysis for laboratory use and especially to stress for the reader the possible errors and misinterpretations.

It is a pleasure to participate in creating this series of 'Laboratory Manuals' in collaboration with Drs. T.S. and E. Work.

The rapid expansion in the field of immunochemistry has necessitated the publication of a new edition of the present manual. Several chapters i.e. those dealing with lectins, HLA-antigens and with the application of crossed immunoelectrophoresis for characterization of membrane proteins, have been extensively revised and extended. Furthermore, new chapters dealing with the complement system, enzyme immuno-assay systems, immuno-fluorescence methods and immunoadsorbent techniques have been added and the recent developments in the methodology

already presented in the first edition have been included.

Cellular immunity has been described as a basis for the use of lymphocytic systems for the tracing of antigens giving rise to delayed hypersensitivity reactions.

Gratitude is expressed to Doctors T.S. and E. Work for their critical evaluation.

JØRGEN CLAUSEN

Copenhagen 1981

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Introduction

1.1. The antigen—antibody reaction

Since the initial discovery of infectious agents and of the acquired defense reactions towards these agents (Pasteur 1876; Von Behring and Kitasato 1890; Koch 1891; Metchnikoff 1892), followed by the demonstration of the presence of specific serum factors neutralizing the infectious agents (Ehrlich and Morgenroth 1900), immunochemistry has developed into a well-defined physicochemical discipline. This subject was initiated by the discovery of precipitating antibodies (Kraus, 1897) and greatly advanced by the discovery of the blood group antigens (Landsteiner and van Der Scheer, 1928). The discovery of the precipitin reaction arose from the demonstration of the precipitate formed when cell-free filtrates of typhoid cultures were mixed with the corresponding antiserum. This technique was made quantitative by Heidelberger and Kendall (1935), thus permitting detection of less than 0.1 μg antigen in solution.

During recent years, precipitation reactions in which antigens and antibodies diffuse through and react in semi-solid matrices (e.g. agar gel) have become essential tools in biochemical analysis. These techniques generally fall into three distinct categories, simple diffusion, double diffusion and immunoelectrophoresis. Before describing them in detail, some understanding is required of the physicochemical factors involved.

1.1.1. Physicochemical aspects of the antigen–antibody reaction

As early as 1902 Arrhenius and Madsen described how the antigen–antibody reaction may be treated on the basis of the law of mass action:

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the combination of antibody (Ab) and antigen (Ag) was considered as a reversible bimolecular reaction:

$$\mathrm{Ab} + \mathrm{Ag} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \mathrm{AbAg} \tag{1.1}$$

where k_1 and k_2 are the rate-constants of the forward and backward reactions. According to the law of mass action, the following relationship holds:

$$\frac{(\mathrm{AbAg})}{(\mathrm{Ab}) \times (\mathrm{Ag})} = \frac{k_1}{k_2} = K \tag{1.2}$$

where (Ab), (Ag) and (AbAg) represent the concentration of reactants and products and K is the equilibrium or association constant. The determination of the value K for any particular antigen–antibody reaction at different temperatures is necessary for determination of thermodynamic constants. The thermodynamic data indicate the nature of the bonds established between the two reactants (see p. 4).

The quantitative nature of the antigen–antibody reaction was first exploited by Heidelberger and Kendall (1929, 1935) who found that it is possible to isolate and estimate the immunoprecipitate formed by adding increasing amounts of an antigen to a fixed amount of antibody. The amount of the precipitated complex increases with rising quantities of antigen up to a certain point. This region of increasing precipitation is referred to as the interval, or zone, of antibody excess. Above this point there is no further increase in precipitated complex as long as some degree of equivalence exists between the two reactants (zone of equivalence). With increasing quantities of added antigen, a so-called zone of inhibition of complex formation is reached (antigen excess): here, there is a progressive decrease in amount of antigen–antibody complex precipitated (fig. 1.1).

These results led to the postulate that the formation of immunoprecipitates is based on a series of bimolecular reactions following the law of mass action:

$$Ab + Ag \rightleftharpoons Ab - Ag$$

$$Ab - Ag + Ab \rightleftharpoons (Ab)_2 - Ag \text{ (antibody excess)}$$

or $$Ab - Ag + Ab - Ag \rightleftharpoons (Ab)_2 - (Ag)_2 \text{ etc.} \quad (1.3)$$

Using light-scattering and fluorescence-quenching measurements, Tengerdy and Small (1966) showed that the primary Ab–Ag complex is established within 10 sec, while equilibrium-dialysis has demonstrated that the final polymerized antigen–antibody complex is formed during the first half hour (Hughes-Jones et al. 1963). Heidelberger's basic concept was further modified by Eisen and Keston (1949) who demonstrated the existence of polyvalent binding sites on the antigen. The antibody–antigen reaction may be considered similar to the binding of drugs to e.g. serum albumin (Klotz 1946; Clausen 1966b).

The antibody–antigen reaction shows temperature dependence of

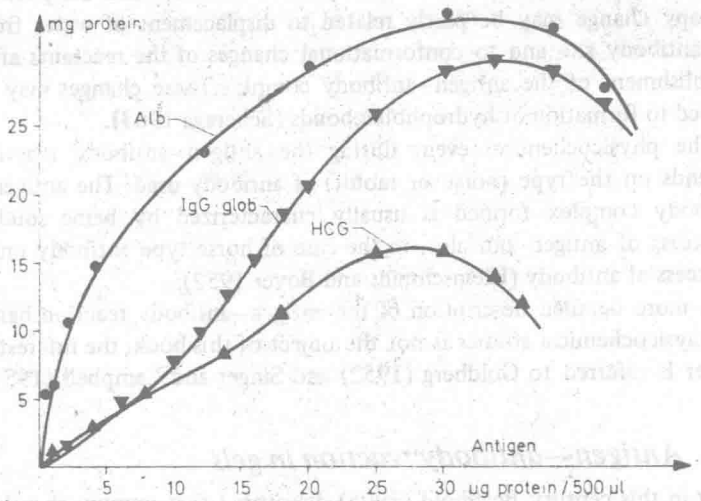

Fig. 1.1. Precipitation curves of three protein antigens. The three curves illustrate the immunoprecipitation in a free buffer solution of human albumin (Alb), IgG globulin (IgG glob) and human chorionic gonadotropin (HCG) with corresponding specific antibodies. *Abscissa:* µg of protein present per 500 µl antigen solution (in 0.9% NaCl). *Ordinate:* mg protein precipitated by corresponding specific antisera per 100 ml solution.

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the rate of association of the two reactants between about 0 to 40°C. Various investigators have found Q_{10} values varying from about 2 (Burnet et al. 1937) to about 1.4. Values intermediate between these extremes may be the most correct. Jerne and Skovsted (1953) demonstrated that the reaction was maximal at a low salt concentration. Finally, the antigen–antibody reaction is dependent on *p*H, showing an optimum at 7.0 (Kleinschmidt and Boyer 1952). The immunoprecipitate is split below *p*H 4.5 and above *p*H 10.0.

From a physicochemical point of view the antigen–antibody reaction is characterized by small changes in the thermodynamic constants. Thus, the change in Gibbs' free energy ΔG^0 seems of the order of –9 kcal/mol. The change in entropy ΔS^0 is positive and the change in enthalpy ΔH^0 is about –9 kcal/mol (Wurmser and Filitti-Wurmser 1957; Karush 1958; see also the survey by Schultze 1962). These values are of the same order as the changes in the thermodynamic constants for binding of drugs to serum albumin (Klotz 1946; Clausen 1966b).

The small changes in the thermodynamic constants and the positive entropy change may be partly related to displacement of water from the antibody site and to conformational changes of the reactants after establishment of the antigen–antibody complex.These changes may be related to formation of hydrophobic bonds (Scheraga 1963).

The physicochemical event during the antigen–antibody reaction depends on the type (horse or rabbit) of antibody used. The antigen–antibody complex formed is usually characterized by being soluble in excess of antigen and also, in the case of horse type antibody only, in excess of antibody (Kleinschmidt and Boyer 1952).

A more detailed description of the antigen–antibody reaction based on physicochemical studies is not the object of this book; the interested reader is referred to Goldberg (1952) and Singer and Campbell (1952).

1.2. Antigen—antibody reaction in gels

Early in this century Bechhold (1905) discovered that immunochemical reactions could also be performed in gels. The 'gel diffusion' method was further developed by Arrhenius (1907) who showed that it could fractionate antigen mixtures such as tetanus and diphtheria toxins in complex with their corresponding antibodies. Qualitative methods for

identification of antigens and antibodies were introduced by Nicolle et al. (1920). Both Petrie (1932) and Sia and Chung (1932) further developed the gel method as a means for identification of bacterial antigens. Later, Maegraith (1933) and Hanks (1935) suggested gel diffusion as a microanalytical tool for demonstration of the antigenic composition of unknown samples.

In 1946 Oudin elaborated the physicochemical and mathematical basis for employing the diffusion in gels of a single antigen and/or antibody for quantitative purposes. The basic work of Oudin (1946; review, 1952) was extended and modified by Ouchterlony (1949) and Elek (1948). In 1953 Grabar and Williams described 'immunoelectrophoresis' which combined the electrophoretic separation of proteins in agar gel with a subsequent antigen–antibody reaction performed by immunodiffusion in the same agar gel. Although the basic principle of this method had been independently developed by Poulik (1952) who combined filter paper electrophoresis with agar gel immunodiffusion, the recent expansion in analytical immunochemistry was initiated by the work of Grabar and Williams. On the basis of their method, Scheidegger (1955) developed his micro-immunoelectrophoretic method which made it possible to separate and identify a large number of antigens in a volume of about 1.5 μl.

Electrophoresis combined with the antigen–antibody reaction was also the basis for the development in recent years of radio-immunochemical techniques (Berson and Yalow 1961; Berson et al. 1964). These techniques have provided the impetus for development of ultramicro-methods for estimation of proteins and peptide hormones in biological fluids (see Chard (1978)).

Gel diffusion reactions are dependent on the high water content (often as high as 99%) of the gelatinous supporting media. This permits the antigens and antibodies to migrate through the gel by diffusion (or be accelerated by an electric field) almost independently of the supporting medium, provided that the diameter of the reactants is less than the width of the microstructure of the gels (Wieme 1957, 1959). Under these conditions the free diffusion of both antigens and antibodies conforms to laws of gaseous diffusion and thus follows Fick's law:

$$dx/dt = D d^2c/dt^2, \qquad (1.4)$$

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