# Immunodiffusion

Alfred J. Crowle

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## SECOND EDITION

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# Preface to the First Edition

In the past decade immunology has enjoyed an obvious rise in popular medical and biochemical thinking, growing from a subject regarded with only moderate interest by the average physician and biochemist of a few years ago to one now often occupying their foremost thoughts. In the author's opinion, there are two reasons for this rise. The first is that allergy is being implicated as a complicating or causative factor in increasing numbers of human diseases, most interestingly those of auto- or isohypersensitization. The second, and that directly relating to the subject of this book, is that by the recent prodigious developments of immunodiffusion serologic techniques, biological research has been provided with a type of analytic tool the like of which in specificity, resolution, and simplicity has never before been known; with it researchers are performing serologic analyses which would have astounded the immunologist of a decade ago. Immunodiffusion as an analytic method has developed from something of a laboratory curiosity, misunderstood and mistrusted, into a well-accepted technique now more often employed by non-serologists than by those who rightly can think of it as a proud development of their own field. Believing that its infancy is passing away and its maturation is beginning, the author thinks that the time has arrived to document basic knowledge of immunodiffusion, formally record the history of its development, demonstrate how usefully it has been employed, introduce its techniques to potential new users, and gather into one reference work various sorts of knowledge on these techniques, often obscure and overlooked, which will aid those who already utilize immunodiffusion.

The theory of antigen–antibody reactions in semisolid media still is rather poorly developed, and its mathematical details will not interest most users of immunodiffusion. Moreover, a discussion of the mathematics of this theory would be excessively lengthy for a book of this size. Hence, theory is approached in Chapters II and III in a general, nonmathematical manner. In Chapter IV, the writer has striven to prepare a compendium of uses to which immunodiffusion has been put, but this summary must be acknowledged incomplete: immunodiffusion now is being applied in so many different fields, often being mentioned only obscurely, that completeness in any such survey is impossible. Chapter V describes in detail principal and accessory immunodiffusion techniques which in the author's opinion will best serve the reader. For those who are already users of immunodiffusion, this chapter includes descriptions

of the latest improvements on established techniques. For the novice, it presents not only general methods but also details on subjects related to immunodiffusion techniques so often hard to find, such as how to photograph or stain antigen–antibody precipitin bands. Appendixes have been composed to supplement this chapter as a handy formulary, and a glossary is appended of terms commonly used in connection with immunodiffusion which might confuse the uninitiated.

The author wishes to thank several of his associates who have contributed to him their most valued assistance in preparing this book: Mrs. Lyle B. McMurry and Mrs. Peggy Braun for their secretarial work; Mr. David C. Lueker who with patience and enthusiasm has set up numerous experiments used to prepare photographic illustrations and to help answer a multitude of technical and theoretical questions which have arisen during preparation of this manuscript; the author's wife Clarice M. Crowle for her encouragement and her faithful help in many particulars, large and small; Dr. James J. Waring for his helpful suggestions on composition. To several others who have participated in lesser extent also goes the author's sincere thanks.

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# Introduction

In the decade since the first edition of this book was published, immunodiffusion has become accepted as a primary bioanalytic technique. More publications mentioning its application now appear in one year than appeared in the decade 1950–1960. Well-standardized and widely employed immunodiffusion diagnostic tests exist.

Perhaps one of the surest signs of its acceptance as a routine tool is its commercialization as evidenced by the concerns developed to market the instruments, materials, and antisera that it employs. Explanatory comments no longer must preface presentation of an immunodiffusion pattern as evidence for the purity or nature of complex macromolecules for the general scientific public now understands and accepts such data. The technique has been essential in following step by step degradation of macromolecules in analyses of their ultrastructure, and then the opposite in their resynthesis. Immunodiffusion is used to monitor manufacturing processes, detect fraudulent products, standardize biologicals, prepare reagents for use with it and with other immunological techniques, classify plants and animals, study the epidemiology of disease, monitor human physiology and pathology, and indicate genetically determined disease risks before the disease develops. It can be used whenever antigens or antibodies need to be quantitated or characterized and, indirectly, to study simpler substances which are neither antigen nor antibody. In its more exotic forms (e.g., two-dimensional single electroimmunodiffusion) it has even become a form of art: students have been known to hang "portraits" of their own serum patterns on walls, and there has been at least one report of discard stained electroimmunodiffusograms disappearing from the laboratory and reappearing elsewhere in the city for sale as a novel form of artistic expression.

The tremendous growth and popular acceptance of immunodiffusion technology during the past ten years has necessitated considerable enlargement and change of this book. It has been written to answer the average user's most common questions: "Can my problem be elucidated by utilizing this technique? Is the substance I am studying an antigen and, if so, what is an antigen? How can I prepare proper antibodies to it? What kind of immunodiffusion test will be the best for me?" Consequently, much more space is used for practical than for theoretical discussion. Whenever possible, the first question is answered by example, and unusual examples have been chosen whenever they have been available because including a chapter solely on applications of immuno-

diffusion techniques, as was done in the first edition, has not been practical. Hence, the examples have been selected to serve a dual purpose: to be illustrative and a general guide to the literature.

The second and third questions concern a large body of information which though essential to is not directly part of immunodiffusion, namely, the nature of antigens and antibodies and characteristics of their interaction in semisolid media. Because this information is not readily found in immunology textbooks or in manuals on general immunologic techniques because of their much broader orientation, an attempt has been made to provide it in Chapters 1 and 2. Especially important, and inadequately discussed in other sources of information, is the question of how to make antibodies for use in immunodiffusion tests. The question is much more complex than it might appear to be on the surface. Different animals make antibodies of differing characteristics; different antigens elicit different kinds of antibody response in a given animal; the nature of antibodies obtained depends upon the time at which they are taken, even within one animal given just one exposure to antigen; the nature of antibodies also depends vitally on how and how much of the antigen is given. Precipitins usually are used for immunodiffusion tests, but if precipitins cannot be raised other kinds of antibodies can be substituted. Antibodies from one species of animal may be better than those from another because, for example, they can be used at high salt concentration for an antigen which is insoluble at low salt concentration. Some species of animal will readily make antibodies to some antigen or antigenic determinant which other species treat as nonantigenic. The precipitins from a horse may be better for qualitative analyses than those from a rabbit and *vice versa*. Whole antiserum may be superior to purified, concentrated precipitins. Points such as these are discussed extensively, because using immunodiffusion to its fullest potential depends on preparing and selecting the best possible antiserum (i.e., analytic reagent) for a given task.

Although antiserum is centrally important in immunodiffusion techniques, other seemingly minor factors may be equally important in determining the outcome of a test. For instance, a reaction can be overlooked for lack of adequate lighting; it may be difficult to photograph; a permanent record of it may be lost because of inappropriate staining and preserving methods. In addition to its extensive discussion of antigens and antibodies, Chapter 2 therefore includes ancillary information necessary to avoid or solve problems such as these. An unusually large number of footnotes appear in this chapter to provide, unobtrusively, valuable explanatory and technical information directly connected with subjects discussed in the text.

In the early days of immunodiffusion, test nomenclature was simply and logically established by the mode in which antigen and antiserum were mixed. Thus, if only one reactant diffused significantly, the technique was called "single diffusion"; both diffused in the "double diffusion" test; and if antigen first was electrophoresed and later analyzed immunologically, "immunoelectrophoresis" was being employed. In the last ten years new ways for intermingling antigen and antiserum have been discovered and exploited. Sometimes their naming has been haphazard; sometimes it has conformed to the descriptive logic of precedent. Chapters 3 through 7 attempt to answer the last question of our average immunodiffusion user ("What kind of immunodiffusion test will be the best for me?") and to explain immunodiffusion techniques in a sequence based on differences in antigen–antiserum mixing techniques. Thus, Chapters 3, 4, and 5 discuss single diffusion, double diffusion, and immunoelectrophoresis, respectively; Chapter 6 discusses the exciting but still relatively little used technique of electroimmunodiffusion; and Chapter 7 describes several even less used techniques in analogous sequence (e.g., two-dimensional single and double immunoelectrophoresis, immunochromatography, immunosedimentation, immunorheophoresis).

The chapter on the history of immunodiffusion is presented last in this edition rather than first as it was in the last edition because today it probably will be of more incidental than essential interest to the average reader and because historical developments in this subject during the past decade are more appropriately described in other chapters to indicate how a technique was conceived of and developed.

The Glossary explains itself. The Appendixes include only technical information deemed essential for general use in immunodiffusion techniques. For example, only the best all-purpose stains for immunodiffusion patterns are included; and only those buffer and electrolyte formulas which are the most efficient, have special utility, or by tacit acceptance have become standards are recorded. This selectiveness does not mean that formulas for stains, buffers, electrolyte solutions, or vehicles of immunization which are not included have all been tested in our laboratory and found inferior or that alternate formulas and techniques are not likely to be useful. Its purpose, as was stated above, is simply to provide the average user of immunodiffusion with the least ambiguous and most useful answers to his problems. One very conspicuous lack in the Appendix is a formulary and list of procedures for special stains and indicators. But this lack is intended because such a formulary could not be prepared adequately without undue use of space and because for these specialized procedures the reader usually will want to refer to an

original description (appropriate references are given in Chapter 2) to understand the rationale as well as technical details of application.

For what interest it may be to the reader, composing the second edition of this book required approximately fivefold more time and energy than writing the first edition. The literature analyzed was larger by equal proportion. Nevertheless, the task has been satisfying and, in the end, inspiring. Surprising as it may seem, my impression is that the most exciting and rewarding immunodiffusion experiments are yet to come; I refer particularly to the tremendous opportunities for new research provided by recent developments in electroimmunodiffusion. My work in composing this second edition has been greatly eased by several individuals to whom I offer my most sincere gratitude, notably my secretary Ada M. Harrison, my wife and literary critic Clarice M. Crowle, and my technical associate Karen S. Jarrett.

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# Contents

# Chapter 1

# Basic Information

The purposes of this chapter are to introduce the reader to the two principal reagents of immunodiffusion tests, antiserum and antigen, and to explain the basic physical events and processes of these tests so as to prepare him for using and understanding immunodiffusion techniques.

## Antiserum

Antiserum is the fundamental reagent of immunodiffusion and provides its great versatility and high specificity. It is used to detect, characterize, and quantitate antigens. Antiserum is produced by animals exposed appropriately to antigen (Weiser *et al.*, 1969). An animal's lymphoid system, recognizing antigen as a foreign substance and an implied biological threat (e.g., infection, tumor), manufactures proteins that react selectively with this foreign "body" and therefore are known as "antibodies." The antibodies used in immunodiffusion tests are most commonly obtained by drawing blood from the antigen-immunized animal, allowing the blood to clot, centrifuging out the clot and accompanying blood cells, and drawing off the clear, amber-colored supernatant antiserum.

### *Antibodies*

Serum contains many kinds of dissolved macromolecules, most of them proteins. These are classified electrophoretically into major groups by increasing isoelectric point (i.e., decreasing electrophoretic mobility) as prealbumins, albumin, $\alpha$-globulins, $\beta$-globulins, $\gamma$-globulins, and basic proteins. In turn, these are subclassified by function, molecular size,

solubility, and antigenic composition (Weiser *et al.*, 1969). Most antiserum antibodies are γ-globulins termed, collectively, "immunoglobulins" because of their functions, and referred to by formula as "Ig" with a following letter to indicate class (e.g., IgA: Weiser *et al.*, 1969; Smith, 1966). Antiserum contains several different classes of immunoglobulin which may or may not be antibodies against the immunizing antigen. Those that are antibodies usually are heterogeneous, differing in how they react with the same antigen and in what effect they consequently produce.

Solubility in distilled water distinguishes between two major varieties of immunoglobulin. Those precipitating when dialyzed against distilled water are euglobulins; those remaining in solution are pseudoglobulins (Boyd, 1966). Against protein antigens, rabbits produce primarily euglobulins, whereas horses form principally pseudoglobulins. But each species of animal also can, and usually does, produce small amounts of the other type of antibody (Siskind, 1966; Johnston and Allen, 1968). The principal euglobulin antibodies are electrophoretically classed as $\gamma_2$- or $Ig_2$-globulins because they are more cathodic than the pseudoglobulins, which, correspondingly, are $\gamma_1$-globulins. Though obsolescent, the terms euglobulin and pseudoglobulin remain useful for indicating whether or not antibodies can be used in distilled water, which may be important in some immunodiffusion tests.

Different classes of antibody molecules share many chemical, physical, and biological characteristics. But because of differences in amino acid constitution they can be distinguished from each other immunologically (Abramoff and La Via, 1970). For instance, rabbit antiserum specific for one class of human immunoglobulin will not cross-react with another class of human immunoglobulin. By this and associated criteria, characterized human immunoglobulins have been classified as IgG, IgM, IgA, IgD, and IgE (Abramoff and La Via, 1970). Such nomenclatural systematization for immunoglobulins is relatively recent. Although it is being applied to antisera of lower animals as quickly as data accumulate and are interpreted, most classes of lower animal immunoglobulins have not yet been identified with their human serum counterparts. Consequently, other interim designations for antibody classes which only suggest similarities to human serum immunoglobulins are frequently used. For example, a 19 S animal immunoglobulin may be called $\gamma_{1M}$ because it is a macromolecular γ-globulin with a $\gamma_1$ mobility in the immunoelectrophoretic pattern for that animal's serum. But it should not be called IgM without considerable proof of its homology with human serum IgM.

The term "19 S" above refers to the physical characteristic of molecular size as estimated by ultracentrifugal sedimentation, in which "S"

signifies "Svedberg" (Boyd, 1966). The class of antibody most frequently used in immunodiffusion tests is an antigen-precipitating 7 S immunoglobulin (precipitin) of molecular weight approximately 175,000 (Tran Van Ky *et al.*, 1966a; Remington *et al.*, 1962). Other classes of antibody may be larger because of attached accessory structures (e.g., 11 S secretory IgA in man, of molecular weight 400,000; Dayton *et al.*, 1971), because they polymerize (e.g., 11 S and 14 S chicken precipitins: Kubo and Benedict, 1969; Van Orden and Treffers, 1968a; Hersh and Benedict, 1966), or because they are manufactured by the body in pentamers (e.g., 19 S IgM antibodies of molecular weight 900,000: Smith, 1966; Wahl *et al.*, 1965; Abramoff and La Via, 1970). Occasionally, biologically active pieces of antibody also may be encountered—for instance, in urine (Remington *et al.*, 1962) or in cattle antiserum (Cowan, 1966b). For immunodiffusion these molecular size distinctions are important, both because of correspondingly different rates of antibody diffusion and because of associated contrasts in reactions with antigen (Paul and Benacerraf, 1966). In recent years the ultracentrifuge has given way to simpler, less expensive ways of estimating antibody molecular size, such as measuring absolute or relative rates of diffusion through agar gels, determining diffusion-limiting pore size in semisolid media, or measuring gel-filtration $R_f$ values (see Chapters 6 and 7).

By definition, all antibodies must be able to complex specifically with antigen. But antibodies differ in effects produced by such complexing and in conditions required for development of these effects. Indeed, they are known by their effects as precipitins (precipitate dissolved antigens), agglutinins (aggregate and sediment suspended antigens), complement-fixing antibodies (on combining with antigen they fix and activate enzymatic serum proteins known collectively as "complement"), opsonins (they combine with particulate antigens to facilitate their phagocytosis), and blocking antibodies (they interfere with manifestations of other kinds of antibodies). Antibody activities need not correspond with antibody immunoglobulin classification, since different classes of antibody may produce similar reactions with antigen. For instance, both $\gamma_M$- and $\gamma_G$-globulins in an antiserum can be precipitins (Pike, 1967; Tran Van Ky *et al.*, 1966a). On the other hand, a given antiserum will be likely to contain various antibodies with differences in both effect on and avidity for the same antigen (Carter and Harris, 1967; Boyd, 1966; Abramoff and La Via, 1970), and among these, individual antibodies will differ as to the portions (determinants) of the antigen with which they combine (Weiser *et al.*, 1969). These different kinds of antibody in a single antiserum, with their individual variations in relation to one antigen, and their competitive (Fiset, 1962; Christian, 1970) or

complementary (Carter and Harris, 1967; Moore, 1961) interplay with each other through combination with the same antigen, define the overall antibody activity of the antiserum (Klinman *et al.*, 1966). As will be seen below, this total activity also is affected importantly by other nonantibody constituents of the antiserum.

Immunodiffusion tests most commonly use precipitins. But in some, antibody complexed with antigen forms clear or "negative" precipitin bands in agar gels, instead of opaque ones (Moore, 1961; Silverstein *et al.*, 1958); and there are immunodiffusion tests that detect blocking antibodies (Patterson *et al.*, 1964a), complement-fixing antibodies (Milgrom and Loza, 1966; Paul and Benacerraf, 1966), agglutinins (Milgrom and Loza, 1967), and antibodies that form no more than primary complexes with antigen (Freeman and Stavitsky, 1966; see Chapter 7 for additional examples). The following discussion centers on precipitins because of their primacy in immunodiffusion. The characteristics and uses of nonprecipitating antibodies in this technique will become evident partly as a by-product of this discussion and partly with later description of specific tests using these antibodies.

### Precipitins

Precipitins are antibodies that insolubilize antigen; hence, an antiserum that produces a precipitate when mixed with antigen solution contains precipitins. But this precipitating capacity for an antiserum is the product of complex agents and events including nature of antibodies, interaction between antibodies, interplay with nonantibody serum constituents, physicochemical conditions, and nature of antigen. Consequently, only a functional definition of precipitins is possible, although most frequently these antibodies are 7 S $\gamma$-globulins that are divalent and have a high affinity for antigen.

Precipitins can be 30 S (Cowan, 1966b), 19 S (Josephson *et al.*, 1962; Cowan and Trautman, 1965; Pike, 1967), 14 S (Orlans *et al.*, 1961), 7 S (Siskind, 1966), or even 4.5 S globulins (Cowan, 1966b). Their electrophoretic mobility depends on the species of animal making them, on the antigen inducing them, and on the immunization protocol employed (Christian, 1970). Rabbits tend to make $\gamma_2$-globulin precipitins (Siskind, 1966); horses more copiously make $\gamma_1$-globulin precipitins (Johnston and Allen, 1968); precipitins frequently occur in both electrophoretic classes of globulin in guinea pigs (Wilkerson and White, 1966) and mice (Krøll, 1970) and occasionally also in man and monkeys (Hillyer, 1969). Guinea pigs injected with foot-and-mouth disease virus produced, within 4 days, 19 S $\gamma_1$-globulin precipitins which could neutralize virus but not fix

complement; but after 15 days they had ceased production of this antibody and instead were manufacturing 7 S $\gamma_2$-globulin precipitins which could both fix complement and neutralize virus (Cowan and Trautman, 1965; Graves *et al.*, 1964). Seven days after infection with the same virus, cattle were making precipitins of 19 S and 30 S $\gamma_1$-globulin, but later they made predominantly 7 S and 4.5 S $\gamma_1$- and $\gamma_2$-globulin precipitins (Cowan, 1966b).

Precipitins are called R- or H-type according to how they precipitate antigens (see section on antigen–antibody precipitation, below); and they can be either pseudo- or euglobulins. But production of one or the other of these types is not an exclusive characteristic of just certain species of animals. For example, conventionally immunized rabbits produce pseudo- and euglobulins, and both R- and H-type antibodies (Siskind, 1966). Horses make predominantly R-type precipitins early after immunization with protein antigens; only later do they produce the predominantly H-type precipitins for which they are renowned (Klinman *et al.*, 1964; Johnston and Allen, 1968). Both R- and H-type antibodies in the horse are 7 S globulins (Allen *et al.*, 1965).

Most precipitins are either $\gamma_1$- or $\gamma_2$-globulins, but $\alpha_2$-globulin precipitins have been observed in rabbits (Strejan, 1965) and horses (Korngold and van Leeuwen, 1962). Precipitins may (Klinman *et al.*, 1966) or may not fix complement (Dupouey, 1963; Cowan and Trautman, 1965; Cowan, 1966b). Although they tend to have high affinity for antigen, this property alone does not make an antibody a precipitin; nonprecipitins can have equally high association constants (Klinman *et al.*, 1966). Both combining sites on an antibody molecule must be free to complex independently with antigen for it to be a precipitin, since if it is not thus functionally divalent not only will it fail to precipitate antigen, but also it may interfere with other precipitins to the same antigen (Klinman *et al.*, 1964). The ability of an antibody to precipitate antigen sometimes is related to its molecular charge, high isoelectric point favoring precipitating capacity (Carter and Harris, 1967). Since delipified antibodies retain normal affinity for antigen molecules but lose their capacity to precipitate them, antibody-bound lipids probably are required to stabilize the forming lattice of antigen–antibody complexes for visible precipitation (Cline, 1967; Tayeau and Jouzier, 1961b).

Certain antibodies in an antiserum must cooperate to precipitate antigen; others may enhance precipitation but not be necessary; still others interfere with precipitation. No single precipitin can recognize more than one kind of determinant on an antigen molecule. Consequently, different precipitins recognizing two or more antigen de-

terminant sites are required for antiserum to precipitate an antigen (Weiser *et al.*, 1969).[1] Interestingly, this can account for the fact that rabbits immunized with too much human serum albumin produce antibodies but not precipitating antisera to the albumin. Having been made partially tolerant to this antigen, they make precipitins that are reactive with too few antigenic determinants to develop a visible aggregate (Christian, 1970).

Nonprecipitating antibodies can aid precipitins by "coprecipitating" with antigen and thus adding stability and bulk to the antigen–antibody lattice. For instance, certain chicken antisera contain two kinds of antibody, one of molecular weight 600,000 which will precipitate antigen alone, and the other of molecular weight 180,000 which is unable to precipitate antigen without help from the first. Both contribute to antigen precipitation by the whole antiserum (Orlans *et al.*, 1961). Some rabbit antisera, in addition to $\gamma_G$-globulin precipitins, contain also $\gamma_A$-globulin nonprecipitins which coprecipitate to enhance antigen aggregation by the precipitins. Both antibodies have similar affinities for antigen, but the coprecipitin is more hydrophilic and has a higher negative charge, and alone cannot form large antigen–antibody lattices (Carter and Harris, 1967).

The proportion of contrasting types of precipitin in an antiserum helps to determine its overall precipitating characteristics. Consider horse antiserum to human $\gamma$-globulin. Both $\gamma_1$ H-type and $\gamma_2$ R-type antibodies are present, but the former usually outnumbers the latter. Since the former has a high negative charge and is more hydrophilic, its resultant characteristic of precipitating antigen over a narrow range of proportions is also characteristic of the horse antiserum itself. But since this characteristic is influenced by the presence and quantity of the R-type antibody in the antistrum, in extremes, such as within an early-bleeding antiserum, horse antiserum can have R-type antigen-precipitating characteristics (Johnston and Allen, 1968).

In some antisera, nonprecipitins may compete with precipitins for antigen determinant sites and, instead of aiding, thereby prevent or diminish precipitation. For instance, some canine antisera to bovine serum albumin contain nonprecipitating antibodies which, when mixed with or used adjacent to canine or rabbit precipitating antisera in immunodiffusion tests, prevent these from precipitating the antigen (Patterson *et al.*, 1964a). Early-bleeding horse antisera can exert a similar effect,

[1] Most soluble antigens (e.g., albumins) are multivalent but have no more than one of each determinant site; only antigens with large repeating units (e.g., $\gamma$-globulins) will have two or more of one kind of determinant site (Pressman, 1967).