ADVANCES IN PROTEIN CHEMISTRY

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PREFACE

The reader who is familiar with the previous volumes of this series will notice some unusual features in Volume XIII. Only four reviews are presented, three of which are long, the last two being among the longest articles that we have ever published. The fact that three lengthy reviews are presented in the same volume arises by accident rather than by design.

In the short opening article Pierre Grabar discusses some important immunochemical methods in their relation to protein chemistry. Since the author himself has been a major contributor in this particular field he writes with intimate personal knowledge of all the problems involved; although the work of many others is discussed, this article is not intended to provide a broad general survey of the field, but rather to give a critical evaluation and offer new perspectives.

In the second review, Bettelheim-Jevons gives a comprehensive discussion of protein-carbohydrate complexes. Because this subject has not been reviewed in these pages since publication of the article by Karl Meyer in Volume II, this review fills an important need in giving a critical survey of progress in this important and difficult field.

A review of the silk fibroins is long overdue. That presented here by Drs. Lucas, Shaw, and Smith of the Shirley Institute in England covers many aspects, chemical, physical, structural, technical, and comparative, and must of necessity be longer than usual.

The final contribution by Katchalski and Sela on the synthesis and chemical properties of poly-α-amino acids deals with a subject which has shown enormous expansion since it was last reviewed in these pages in 1951. It is of the greatest interest to chemists and biochemists both for the significance of the polyamino acids themselves and for the light which they can throw on our understanding of the properties of proteins. The unusual length and comprehensiveness of this review therefore appear justified by the scope of the subject.

The editors trust that the comprehensiveness of these reviews will make them standard references.

Originally it was planned to include in this volume an article by Dr. Howard K. Schachman on "Ultracentrifugation in Biochemistry." Dr. Schachman responded to our invitation by preparing an admirable review which has grown in the course of the last year into a comprehensive monograph. This is so extensive, and we believe will be so important for users of the ultracentrifuge and others, that the publishers have decided, with our

approval, to issue it as a separate monograph, independent of "Advances in Protein Chemistry." The editors wish, however, to express their pleasure in the fact that they supplied the original stimulus which led to the writing of this monograph.

The organization of the present volume is not intended as a model of the future issues of *Advances*. In the future we plan to publish volumes containing a greater variety of articles, usually seven or eight in each volume, most of which will be shorter, probably of the order of 30–60 pages. Occasionally an important subject may deserve a more comprehensive review of perhaps 100 pages. It will remain our general aim, however, to provide thoughtful and critical articles, covering limited but significant fields, written by authors who are intimately familiar from personal experience with the subjects which they discuss.

November 1958

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CONTENTS

THE USE OF IMMUNOCHEMICAL METHODS IN STUDIES ON PROTEINS

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I. Introduction

In writing this article, the author's aim is not to review the results obtained in studies on proteins by immunochemical methods but to try to inform the reader of the possibilities which these methods can offer to the protein chemist. To illustrate these possibilities and their limitations, a certain number of results will be quoted, most of them from work done in this laboratory. No attempt has been made to survey the literature on this subject, which becomes more and more extensive. However, some authors have either not taken full advantage of the possibilities of the methods used, or they have overlooked some of the pitfalls or disadvantages of these methods. In other cases, one often sees publications in which a problem could have been resolved more easily or decisively by immunochemical methods than by others. Thus the presentation of this subject seemed justified to the author, and he hopes that it will prove useful.[1]

[1] The main lines of this article were presented as the First Michael Heidelberger Lecture in Immunochemistry at the College of Physicians and Surgeons, Columbia University, New York City on January 19, 1957.

II. The Immunochemical Methods

The main value of immunochemical methods is that they are specific and sensitive. It is also known that the specificity of the antigen-antibody reaction is due to the spatial configuration of some chemical groupings in the antigen molecule. However, the dimensions of the "antigenic motive"[2] of a protein antigen are not yet established with certainty. We know (see below) that several determinant groupings can exist in one molecule, and one can suppose that antibodies may possess specificities corresponding to more or less large areas of these patterns. In other words, some antibodies would react specifically with a larger surface of the antigen molecule than others. This would explain the fact that in cases of cross reactions only a part of the antibodies for the homologous antigen reacts with a heterologous molecule.

For the moment, however, the hypothesis cannot be excluded that the antibodies which react only with the homologous antigen are specific for a small "antigenic pattern," which is absent in the structure of the heterologous antigen. If this hypothesis were correct, then the heterogeneity of the specifities of antibodies in an immune serum would not be due to a more or less large "antigenic motive" on the surface of the antigen, but to a more or less large number of different antigenic determinants in its structure. Some investigations of this question have been made with artificial antigens, i.e., with proteins coupled with known small molecules, but no precise information seems to exist for natural antigens. One must add, however, that the absence of precise knowledge on this point is of relatively minor importance for the following discussion.

The sensitivity of various immunological methods is in general very great but may vary considerably from one method to another. If, for example, it is possible to observe a positive precipitin reaction with a few μg. of antibody per ml., a passive hemagglutination reaction can be obtained even with concentrations a thousand times smaller. In this article, only the precipitin reaction will be considered, because it is directly visible and can be studied by a quantitative chemical method in absolute weight units, and because it is the only one which can be used in gelified media. This reaction has the disadvantage of being limited to cases in which the antigen is in aqueous solution.

If insoluble antigens have to be studied, some limited information can be obtained by the use of certain indirect methods, such as complement fixation, the fluorescent antibody technique (9), or the antiglobulin consumption test (85). Other methods (passive hemagglutination, anaphylac-

[2] The "antigenic motive" of a molecule is defined as the sum of the determinant groupings which are responsible for the antigenic specificity of this molecule.

tic reactions, etc.) may also occasionally give useful information, but they have not yet been extensively applied to studies considered in this article.

The quantitative determination of specific precipitation by the method of Heidelberger and Kendall (35) allows the establishment of the so-called "precipitation curves," and equations for these curves may be derived. This question has been reviewed by Treffers in the first volume of this series (86); thus, it need only be recalled that two characteristic shapes of these curves are known: the rabbit-type which has no prozone and the horse antiprotein type which is bell-shaped. Numerous investigations have been made by means of this quantitative method. The principal additions to knowledge which can be obtained with it are: quantitative estimation in units of weight of the antibodies in an immune serum or of an antigen in an unknown solution, control of the purity or the homogeneity of an antigen preparation (to a certain extent, see below), information on the degree of cross reactions between two antigens or a native and a modified antigen. Qualitative tests made on the supernatants also give useful supplementary informations.

The use of gelified media for the precipitin reaction was introduced in 1905 by Bechhold (1) and since that time several authors have described experiments made in gels, in order to stabilize the so-called "ring test," which should be called the "disk test" because it corresponds to the formation of a precipitate at the interface of two liquids, one containing the antigen and the other the antibodies. Thus, for example, in 1920 Nicolle *et al.* (63) described a quantitative estimation of toxins using a toxin antitoxin flocculation reaction in a gel.

Better knowledge of the principles governing the precipitin reaction allowed a development, in recent years, of several methods of specific precipitation in gels. Ten years ago, using tubes with agar gels, Oudin (65) established his simple diffusion method. Independently, Ouchterlony (64) worked out a double diffusion method using agar plates in Petri dishes. In the simple diffusion method, the antigens, used in excess, diffuse into the gel containing the antibodies and form specific precipitation disks or zones. The number of such disks is less than, or equal to, the number of independent precipitation systems (i.e., antigen-antibody reactions) present in the mixture examined.

The double diffusion method in agar plates allows not only the determination of the number of precipitating systems but also the comparison of antigens present in different solutions. In the original Ouchterlony technique, troughs are made in the agar gel which are filled, e.g., one with the immune serum and two others with two different solutions containing the antigens. Figure 1 shows three possible patterns: (*1*) identity of antigens when the precipitation line is continuous; (*2*) cross-reacting antigens when

a spur appears at the junction of two lines; and (*3*) different antigens when the lines cross without junction (51).

Several variants or modifications of these methods have been described, some of them very ingenious or adapted to particular studies (for more details and references see, e.g., (20, 62). These techniques have already been used by many investigators and have produced useful information. The double diffusion technique can also furnish interesting results for the analysis of the supernatant solutions of precipitation curves, particularly in cases in which the antigen solution contains more than one substance. The points of equivalence for each of the constituents can be roughly estimated (Fig. 2).

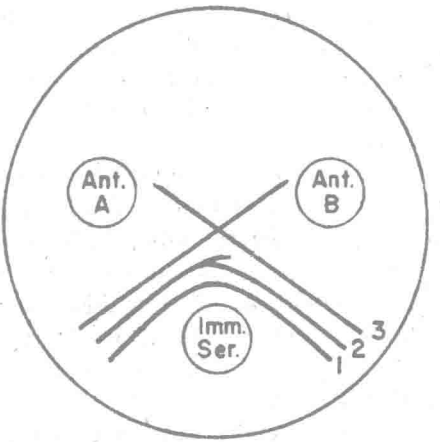

FIG. 1. Ouchterlony's double diffusion method. The troughs contain the immune serum (Imm. Ser.) and two different solutions of antigens (Ant. A and Ant. B); line 1, identity of antigens; line 2, cross-reacting antigens; line 3, independent reactions.

The use of these methods, however, is somewhat limited for three reasons: (*1*) Two lines or zones may be confluent, which is sometimes difficult to recognize. (*2*) In some cases, mainly in the presence of a large excess of one of the reactants, a line or zone given by a single antigen-antibody system can separate into several lines (Fig. 3), as has been shown by Burtin (3), by Kaminski (45, 72), and recently by Jennings (39). The reason is not yet clear; it is possible that we are dealing with a phenomenon of the same kind as the Liesegang rings, but it may be due to the heterogeneity of antibodies against the same antigen. Anyhow, the possibility of such a phenomenon must not be overlooked, and in some cases it is not easy to distinguish a doubling of a zone from two or more confluent zones. (*3*) The third difficulty is encountered with complex mixtures. If the number of individual lines is large, it becomes very difficult to identify each line

and define the constituents. When every one of the constituents is available in a pure state, it is possible to identify the different lines given by the mixture by means of Ouchterlony plates. But such cases are not frequent, and generally only a few of the lines can be identified.

In order to overcome these difficulties and to be able to define or identify all the constituents, a new physicochemical method called immunoelectrophoretic analysis has been elaborated (29). In this method, the mixture

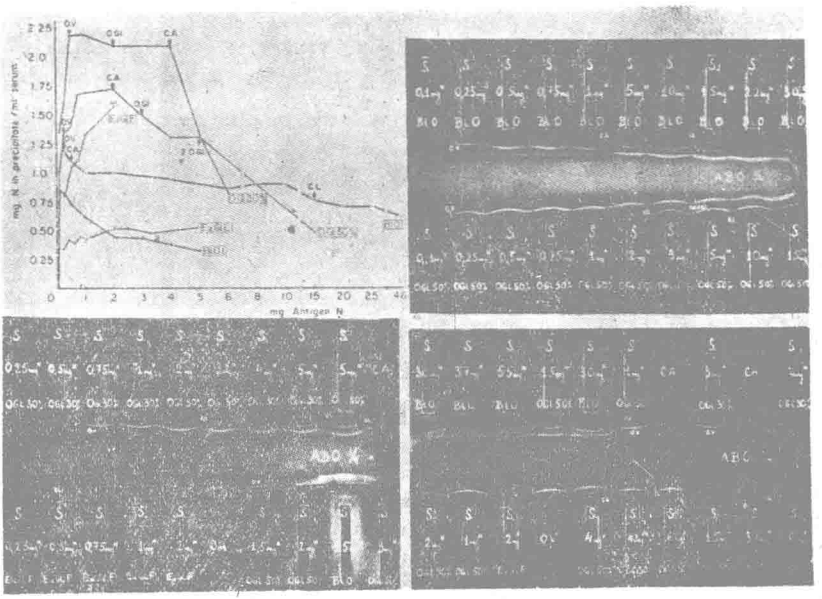

FIG. 2. Double diffusion in agar used as control of the supernatants (*S*) in the establishment of a precipitin curve with a mixture of antigens. *Bl.O.*, total egg white; *O.Gl.* 30% and *O.Gl.* 50%, ovoglobulins precipitated with 30 or 50% saturation of ammonium sulfate; *Eu.Gl.*, euglobulins of the egg white; *Bl.O.l.*, mixture of ovalbumin (*OV*), conalbumin (*CA*), lyzozyme, and ovomucoid (*OM*); *ABO*, rabbit anti-total egg white antiserum (44).

is first submitted to electrophoresis in a transparent gel. This is generally agar, but some other substances, like pectin, can also be used (27). At the end of the electrophoresis, the components of the analyzed mixture have been separated by the electrical current in the gel and occupy different positions, depending on their relative mobilities. An immune serum, rich in precipitating antibodies, is then poured into elongated troughs made in the gel and parallel to the axis of migration. The antibodies diffuse perpendicularly to this axis and, as in the double diffusion method of Ouchterlony, when antibodies and antigens meet in suitable proportions, there will be specific precipitation in form of arcs (Fig. 4).

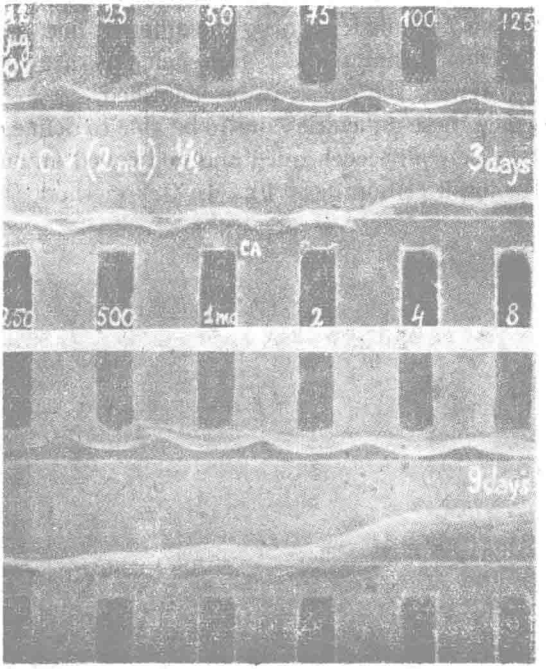

FIG. 3. Dissociation (splitting) of specific precipitation bands as a function of time, in the antigen and antibody excess zones of a single antigen-antibody system, but in the presence of a small quantity of an impurity; *CA*, conalbumin; *OV*, ovalbumin; *AOV*, rabbit antiovalbumin immune serum. The equivalence zone corresponds to 100 μg. *OV* (45).

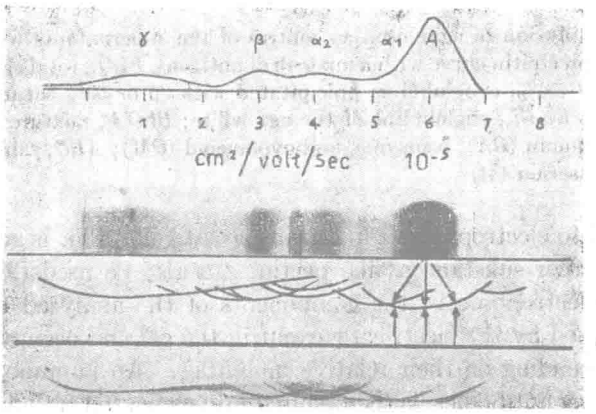

FIG. 4. Principle of immunoelectrophoretic analysis as applied to human serum compared with a free electrophoresis diagram, simple gel electrophoresis with colored bands, and, at the bottom, a photograph of an immunoelectrophoretic analysis. The arrows show the direction of antigen and antibody diffusion (29).

The development of these arcs can be followed in time by simple contact photography and when the process has proceeded sufficiently, the gel can be washed and then dried at 37°C. A wet filter paper is applied to the gel surface to prevent cracking. The gel is transformed into a thin transparent film, and the lines of specific precipitate can be colored by different histochemical reagents (91, 93).[3]

In this way, some of the antigens can be characterized by their chemical nature, such as, presence of lipids, carbohydrates, or particular metals, as well as enzymatic activity, e.g., (87, 88).

The main advantages of the immunoelectrophoretic analysis are: (*1*) its simplicity; (*2*) the possibility of analyzing even very complex mixtures; (*3*) each of the components can be defined or identified by its electrophoretic mobility. Since the gel is very rich in liquid (98.5–99 %), the electrophoretic mobility is nearly the same as in a liquid, and adsorption by the agar has so far been found in the case of only one substance, lysozyme (44). It is, however, still possible that the migration of some very large molecules will be slowed down by the structure of the gel (95). The material of the gel, such as agar, possesses a certain electrical charge, and, therefore, an electroendosmotic flow of the liquid in the gel is provoked by the electrical field. But this flow seems not to interfere with the individual mobilities of the constituents of different mixtures so far studied and, in general, when relative mobilities in agar gels and in liquid media of known substances were compared, the results obtained were similar (97).

Although absolute mobilities in the gel can be calculated, the present author thinks that there is no particular interest in this determination, because the conditions are arbitrarily chosen (concentration of the agar, ionic strength, nature of the buffer solution, etc.). However, it is important to check the relative mobilities of the components of a mixture, particularly, when they can be related to the absolute mobility of a known substance under standardized conditions in liquid medium (Tiselius method). In order to calculate these relative mobilities, due account must be taken of the electroendosmotic flow (20). The simplest way to determine the zero-migration point is to perform simultaneously an experiment with a substance which does not migrate at all, but which will be transported only by electroendosmotic flow. So far, the best results were obtained by using dextrans or levans of a mean molecular weight of 50,000–150,000. These substances can be colored in the gel after the electrophoresis, and the center of the resulting band is taken as the point of zero migration (Fig. 5) (89). If substances of smaller molecular weights are used, the band will be larger because of free diffusion, and precision is lessened.

[3] A microtechnique of immunoelectrophoresis has been described by Scheidegger, which can in certain cases present some advantages (73).

III. The Principal Limitations of Immunochemical Methods

The advantages as well as the limitations of immunochemical methods are due to the use of a specific reagent, the antibody.

In Volume XII of this series, Isliker has reviewed the question of anti-

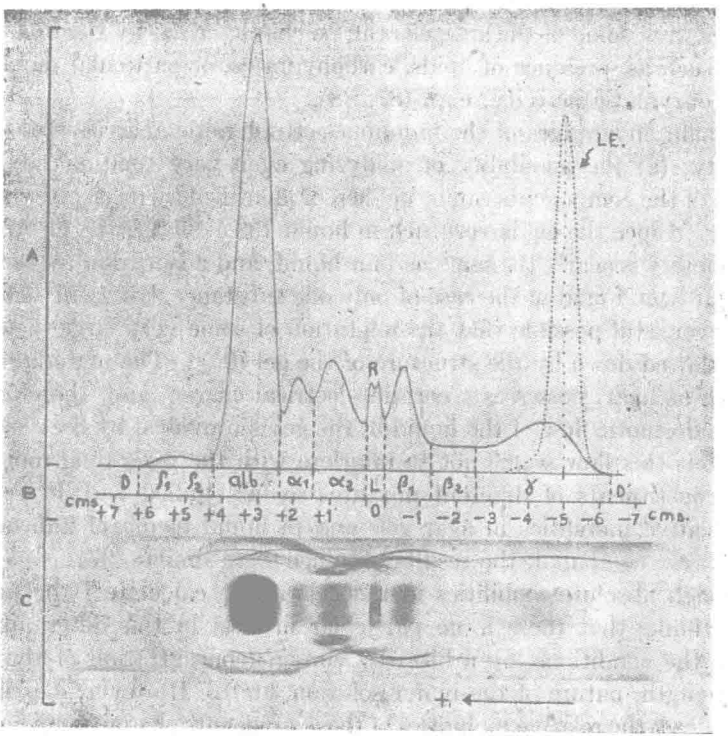

Fig. 5. Quantitative estimation (*A*) of colored bands obtained in simple gel electrophoresis (*C*) as compared to the qualitative distribution of the various constituents of a human serum by immunoelectrophoretic analysis and to the displacement by electroendosmosis of a preparation of levan (*LE*); the distances (*B*) from the starting point (*R*) are expressed in cm. (89).

bodies (37). In order to avoid repetition, this article will deal only with the aspects of this problem which have a direct connection with the use of antibodies as reagents in the precipitin reactions.

Because these reagents are formed in animals receiving injections of antigens, the main problem is how to provoke the formation of precipitating antibodies in a conveniently chosen animal at a maximal concentration. Lack of precise knowledge of such questions as the antigenicity of a substance, or the mechanism of the formation of antibodies, obliges the

immunologist to proceed empirically, and nearly every laboratory uses somewhat different procedures of immunization.

Two cases may be envisaged: (*1*) If the aim is to obtain a particular antibody in high concentration, which will be the case when quantitative precipitation techniques are to be used, it will be advantageous to make the injections with a purified antigen. But, as it is only rarely that such an antigenic preparation does not contain small amounts of some other substances possessing antigenic properties, the immune serum will contain antibodies directed against these impurities, and their amount may be large if the impurity is a highly antigenic substance. Control experiments should be run in order to verify the nature of the antibodies in an immune serum, and in some cases one is obliged to absorb from such sera the undesirable antibodies by suitable preparations of the expected impurities. (*2*) In other cases, immune sera are prepared for the study of mixtures or for the control of purity. At first sight this seems simple, but in practice difficulties are encountered due, on the one hand, to differences in the antigenicity of various components of the mixture and, on the other hand, to variations in the response of the injected animals.

The exact reasons for the antigenicity of a substance are not yet well established. In general, it is admitted that proteins are antigenic, but it is well known that some are completely devoid of this property or are very poor antigens. Gelatin, for example, has been considered as nonantigenic; we know now that it is an antigen, but of low potency (60). This could be explained by saying that gelatin is a degradation product, but we know that some degradation products, such as the first products of enzymatic cleavage of serum albumin, are antigenic (56). Another example of a poor antigen is hemoglobin, although it is a native undegraded protein. These differences of antigenicity among proteins imply that particular care must be taken in the preparation of immune sera in order to obtain, if possible, antibodies directed against all the proteins present in the injected mixture. In practice, the simplest way is to hyperimmunize the animals in the hope that, in the end, all of the antigens will provoke a response. The use of different animal species for the production of immune sera may be of value.

The other difficulty arises from the individual variations in the response of animals to immunization. Most of the animals used currently for the production of immune sera, i.e., rabbits, goats, and horses, are not inbred strains[4], and their responses may vary within wide limits. Figure 6 shows results obtained by the immunoelectrophoretic method with several immune sera of different origin. Notable differences can be seen; in most cases, the differences are mainly quantitative, i.e., the quantity of anti-

[4] It seems that more regular results can be obtained by the use of pure breed chickens for immunization (98).

bodies against one of the antigens differs from one animal to another. But in many cases qualitative variations can be observed, i.e., one of the animals has not formed any antibodies at all for one or other of the antigens. In a series of 20 rabbits identically immunized with normal human serum, 1 or 2 did not form antibodies, e.g., for serum albumin or for γ-globulin (Fig. 6D, rabbit No. 805), while the others have produced these antibodies (Fig. 6E, rabbit No. 810). It is therefore very important to immunize a

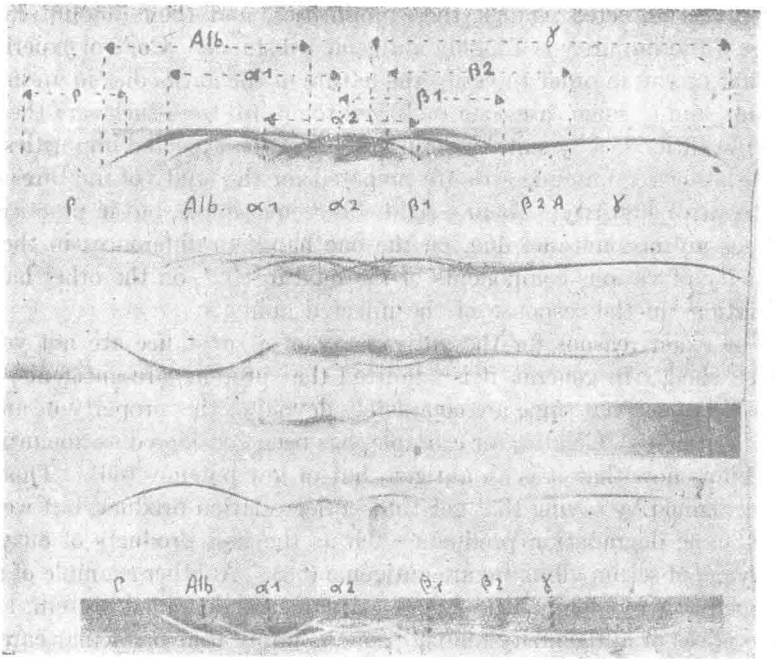

FIG. 6. Immunoelectrophoretic analysis of normal human serum developed with immune sera from different animals. (*A*) mule No. 282; (*B*) horse No. 31; (*C*) horse No. 1299; (*D*) rabbit No. 805; (*E*) rabbit No. 810; (*F*) duck No. 4 (14).

number of animals, to check every individual serum, and then to make a pool of the best sera so as to have a maximum number of different antibodies in the mixture. Such a procedure has been adopted for the horse immune serum against human serum, which is now produced by our Institute. Such sera have been used in most of our investigations on the components of human serum (Fig. 7).

Even when these precautions are taken, it is not absolutely certain that the resulting immune serum will contain antibodies against all proteins of the injected mixture, and in the interpretation of experimental results it is wise to take account of this possibility.