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Immuno Enzyme Techniques in Cytochemistry

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verlag chemie
Weinheim
Deerfield Beach, Florida
Basel

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Publisher's editor: Dr. Hans F. Ebel
Production manager: Dipl.-Ing. (FH) Hans Jörg Maier

This book contains 84 figures and 16 tables

Deutsche Bibliothek Cataloguing-in-Publication Data

Kuhlmann, Wolf D.:
Immuno enzyme techniques in cytochemistry / Wolf D. Kuhlmann. – Weinheim ; Deerfield Beach, Florida ; Basel : Verlag Chemie, 1984.

ISBN 3-527-26078-1

Composition: Mitterweger Werksatz GmbH, Plankstadt
Printing: betz-druck gmbh, D-6100 Darmstadt 12
Bookbinding: Schäffer OHG, D-6718 Grünstadt 1
Printed in the Federal Republic of Germany

Foreword

The inventor of immunocytochemistry, Dr. Albert H. Coons, Professor of Pathology at Harvard Medical School, had as his original intent the identification of putative foreign antigens in affected tissues in certain diseases. It seems appropriate that Dr. Kuhlmann has aimed this treatise at the histopathologists who will continue the exploration of disease states initiated by Dr. Coons and who also will extend these investigations further into the related research areas of virology, bacteriology, and parasitology.

The original procedure of identifying and localizing specific molecules *in situ* in cells and tissues was based on the use of antibodies labeled with fluorochromes. Some of the subsequent milestones in the methodology which are directly related to Dr. Kuhlmann's review include the use of ferritin as a label detectable at the ultrastructural level by Dr. Seymour J. Singer, the use of enzymes with both colored and electron dense reaction products as markers by Drs. G. Barry Pierce and Paul K. Nakane, the development of the histochemical horseradish peroxidase reaction by Dr. Morris J. Karnovsky and its application as an immunocytochemical marker by Dr. Stratis Avrameas, and, finally, the production of the PAP (peroxidase-anti-peroxidase) procedure by Dr. Ludwig A. Sternberger which has greatly facilitated all immunocytochemical investigations. This sequence of events led to an extensive application of the immunoperoxidase technique to biological problems. However, whereas the procedures and results of immunofluorescence have been reviewed extensively, there are no recent reviews of the methodology of the immunoperoxidase techniques. The time seems right, therefore, for such a summary of the concepts and practical aspects of immunoperoxidase localization of biological molecules. This monograph, however, will have even broader use as a handbook for all approaches to immunocytochemistry because of its discussion of the preparation and purification of antigens and antibodies, tissue preservation, control reactions, and the recognition of false positive and false negative reactions.

I am particularly pleased that this monograph is dedicated to Dr. Wilhelm Bernhard. As one of the pioneers in electron microscopy, he was already in 1955 entertaining ideas of applying Coons' method to ultrastructural investigations, and he quickly applied Singer's ferritin marker to his studies of viruses. In particular, during the period when immunoperoxidase methods were refined and applied in his and Dr. Avrameas' laboratories by Dr. Kühl-

mann, among others, Dr. Bernhard rightly perceived the extraordinary potential of immunocytochemistry as a tool which can bridge molecular, cellular, organismal, and clinical investigations.

Prof. Dr. E. H. Leduc
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Preface

This book deals with immunohistological techniques which are sensitive tools for the elucidation of cellular functions in the normal and diseased states. Immunohistological methods are specially applied in biology and medicine and form important bridges between cell biology and pathology.

The book was written for those who seek to understand immunohistology in some depth and aims at a suitable combination of theory and practice. In particular, the preparation of reagents and the methods of sampling are stressed in order to avoid the production of artefacts.

The treatment begins with the general problem of obtaining satisfactory antigen and antibody preparations. Simple methods are described for their qualitative and quantitative evaluation. This is followed by a survey of marker molecules, their conjugation with antibodies, and the purification of conjugates. Methods for histological immunolocalizations and specimen preparations as well as cytological assays for light and electron microscopic immuno-stainings form the main body of the book. Wherever needed the reader is warned of inherent pitfalls.

In handling the vast literature, I have concentrated on key references rather than citing all papers relevant to a given subject.

Several parts of this work were begun when I spent some years with Dr. W. Bernhard, Dr. E. H. Leduc and Dr. S. Avrameas at the Institut de Recherches Scientifiques sur le Cancer in Villejuif/France. To all three I am most indebted for constructive advice pertaining to my own investigations. I very much appreciate the fact that Dr. P. Peschke from my laboratory accepted the invitation to contribute to the chapter on antigen evaluation and purification. Also, I express my gratitude to Mrs. M. Kuhlmann, Mr. H. E. Lehmann and Mr. J. Wiegand for their most valuable technical help. In particular, I wish to thank Dr. E. H. Leduc from Brown University in Providence/USA for her critical reading and help in the composition of the manuscript. I thank the Deutsche Forschungsgemeinschaft for generous support of the experimental studies.

Heidelberg,
June 1983

W. D. Kuhlmann

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Introduction

The introduction of light and especially electron optic systems for morphological studies of cellular and subcellular structures has enabled significant advances to be made in the knowledge of cell biology and normal and diseased organs. However, spatial and temporal aspects of cellular processes and the functional or evolutionary significance of the increasing complexity of higher organisms cannot be elucidated simply by comparing fine structures. In this context, classical histochemistry (reviewed by Pearse [1]) and new specific cytological procedures under development allow relationships between biological structure and function to be more readily discerned.

In order to understand the molecular composition of organs at the cellular level, the combination of immunological and histological concepts is a promising line of research which proves extremely useful for histopathology. Antibodies possess a high degree of specificity towards antigenic determinants. Because of the narrow range of specificity of an antibody molecule to bind with its antigenic determinant, immunochemical methods are part of the most sensitive techniques in molecular biology and biomedicine. With respect to the definition of antigenic molecules (substances which initiate the formation of and react with antibodies are called antigens), immunoserological analyses of organs of normal state and in disease are of great importance. To this aim, qualitative and quantitative approaches have been described since the very early years of this century e.g. by Ehrlich, Landsteiner, Witebsky, Heidelberger, Marrack, Kabat, Oudin, Grabar and schools derived from these pioneers in immunochemistry. In our day, further developments of highly sensitive techniques like those based on radio- or enzyme-immuno-assays are still in progress. The principle of an immunoserological analysis of organs relies on the use of immune sera produced by heteroimmunization of animals or on the use of antibodies produced by hybridomas or on the occurrence of autoantibodies in connection with certain diseases by which the corresponding antigens are detected in body fluids or in organ extracts. A number of phenomena which resemble antibody reaction are shared by lectins. These occur in a variety of plants, invertebrates and vertebrates [2, 3] and are used for the study of carbohydrate moieties in cell components. The latter type of reaction is important per se but is not within the scope of this work.

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The immunofluorescent approach introduced by Coons and co-workers [4, 5] opened specific investigations on cellular structure and function at the light microscopic level. In the

meantime, considerable progress was achieved, and immunofluorescent methods have progressed from pure scientific research towards histopathology [6]. It is evident that analogous techniques could also be useful and important for ultrastructural studies.

In principle, the resolution of the electron microscope enables the demonstration of an antibody molecule which has reacted with its antigen. However, after usual resin embedment single protein molecules in the tissue cannot be identified because such molecule groups are not more electron dense than the surrounding matrix. In consequence, unlabeled antibodies are only suitable for the demonstration of isolated particles when measurable and reproducible changes in density or definite structural changes are obtained [7, 8].

The purpose of most immunohistological procedures is the identification and characterization of cell structure/function *in situ* rather than immuno-staining of physicochemically isolated cell constituents. Hence, the respective immunological ligand must be "labeled" in a characteristic way so that the antigen-antibody complexes become visible. Suitable substances for labeling purposes are those which lead to a distinct color reaction (light microscope) or which give significant deflection of electrons in the electron microscope. A milestone in immuno-electron microscopy was then the conjugation of the metalloprotein ferritin with antibodies [9] which opened a new era of ultrastructure research.

It is now well established that immunological concepts of cellular ligand assays at both light and electron microscopic levels are important for the study of histogenesis, histodifferentiation and histopathology of organs. The aim of the present work is to describe the major steps in preparation of immunohistological reagents on the one hand and in tissue sampling on the other hand.

The detection of intracellular molecules is especially emphasized which is much more intricate than that of extracellular spaces and cell surface membranes. The use of solid tissues instead of single cell suspensions or monolayer cultures is preferentially treated for the reason that tissues or their fragments represent the majority of specimens in histopathology and that, according to current experience, pitfalls are mainly observed with such solid organ preparations. In any case, principles in the preparation of immunohistological reagents are the same and theoretical as well as practical considerations of tissue sampling are quite similar for both tissue fragments and single cells.

When cells are to be studied morphologically, preservation of their structure and minimal alteration from the living state must be ensured. Thus, the adaption of a fixation method is in most cases necessary. However, for immunohistological work, one of the most limiting factors impeding full utilization of immunological reagents is fixation and embedment (e.g. epoxy resins) of biological specimens. Guidelines have been designed to deal with the methodology of light and electron microscopic immunohistology (see references [6, 10, 11]), but immunolabeling of cellular components remained in many cases problematical. Numerous publications during the last decade have shown that by experimental testing and methodological improvement of immunochemical and cytological parameters, conditions can be worked out under which progress in intracellular localization of tissue molecules is obtained.

Cellular labelings by use of horseradish peroxidase as marker become more and more preferred over immunofluorescent (light microscopy) and ferritin labelings (electron microscopy). In the present work, peroxidase techniques and especially the use of peroxidase labeled antibodies in light and electron microscopic immunohistology are described. Numerous data from studies in our laboratory are given. Practical methods as well as theoretical backgrounds are incorporated. Selective procedures are proposed and methodological approaches are

incorporated in the respective chapters. When necessary, more detailed description for practical work is given in Chapter 8. For histopathology, the diagnostic possibilities of immuno-staining are given in Chapter 5.2 in which the gastrointestinal mucosa is chosen as model. A complete histopathological treatise which would include other organs of histopathological interest is not intended.

A general and ideal method for the specific localization of cellular ligands by immunological methods is not available. Procedures for light and electron microscopic immunostainings must be established for each biological model. Immunohistology may be divided into two particular parts, i.e. (a) the preparation of immunohistological reagents and (b) the cytological assay, a division which should be regarded with reservation but which holds true inasmuch as two different areas of research meet together, namely immunochemistry and morphology. Thus, chapters are arranged in such a way as to give (a) basic and practical information on the immunological part and (b) basic and practical information on the morphological part of immunohistology.

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1 Basic Methods and Aspects in Immunological Reagent Preparation

The conditio sine qua non for reliable immunohistology is the use of specific reagents, and qualitative and quantitative evaluations of immunocytochemical reagents are of primary importance. A source of pitfalls is the fact that pure antigen and specific antibody are often not available. Under such conditions, the investigator himself has to prepare both the antigen of interest and the corresponding immune serum. Thus, it is clear that one must be familiar with techniques for the control of antigens and antibodies. Furthermore, principles and practice of standard procedures for the purification of cellular macromolecules should be understood. Knowledge of the latter cannot be presumed from pure morphologists but it is necessary for the reliable production of reagents and the correct assessment of the obtained immunocytological assays. We begin with chapters on analytical methods; preparative procedures follow thereafter. Both can be regarded as basic for serological work in the field of immunohistology. In this context, theoretical and practical concepts are considered. When necessary, examples from our research area are included for explanation. Molecules of interest to us are the oncofetal antigen alpha$_1$-fetoprotein, isolated from amniotic fluid and liver, and acid and neutral glycoconjugates, isolated from gut mucosae. These molecules are taken for the production of specific immune sera which eventually serve for functional and structural studies of gastrointestinal organs by immunohistology.

1.1 Evaluation of Antigens and Antibodies

The introduction of quantitative techniques for the estimation of antigen-antibody reactions by Heidelberger and co-workers [1, 2] began the era of modern immunochemistry. In the meantime, a variety of *in vitro* assays for qualitative and quantitative evaluation of antigens and antibodies have been developed from which the radio- and enzyme-immunoassays are the most sensitive [3–5]. For a complete review of available procedures and extensive biochemical characterization we refer to the respective monographs and handbooks [6–9]. Since we have to consider relevant procedures for immunohistological work, electrophoretic and gel diffusion techniques are of special interest. The latter methods prove very

useful for precipitating antigen-antibody systems with which we are mainly dealing. From the numerous methods available (Table 1 – 1) a selection is presented in more detail.

Table 1-1: Useful and Widely Employed Gel Electrophoretic and Immunological Gel Diffusion Techniques.

Method	Qualitation	Quantitation[a]	Reference
Electrophoresis in agar, agarose	antigens	–[b]	[10]
Electrophoresis in polyacrylamide	antigens antibodies	–	[11, 12]
Single linear immunodiffusion (ID)	antigens antibodies	antigens antibodies	[13, 14]
Double radial ID	antigens antibodies	–	[15]
Single radial ID	–	antigens antibodies	[16]
Immunoelectrophoresis (IEP)	antigens antibodies	–	[17]
Rocket IEP	–	antigens antibodies	[18]
Two-dimensional IEP	antigens	antigens	[19, 20]
Counter-IEP	antigens	–	[21]

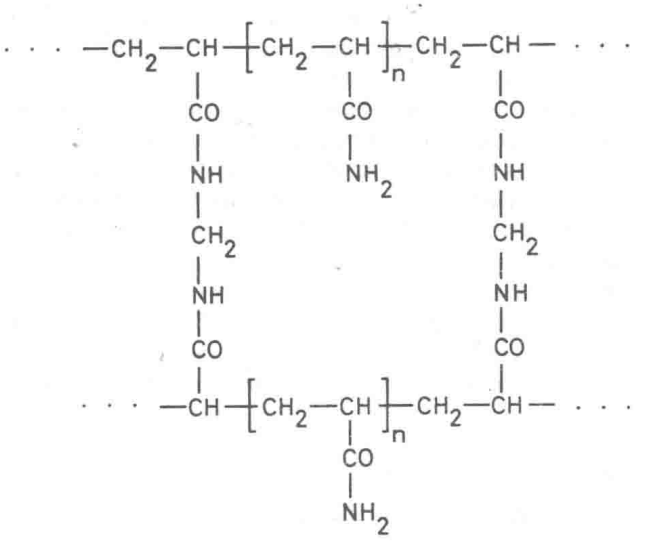
[a] Sensitivity ca. 10^{-5} g/mL; in combination with enzyme labeled antibodies/antigens and by revealing the enzyme activity, sensitivity will give values of ca. 10^{-7} g/mL;
[b] Not principle usage;

1.1.1 Gel electrophoresis

The migration of charged particles in an electric field is the physical principle of electrophoresis. Tiselius [22] was the first to make use of the observation that proteins behave in aqueous solution as charged colloids. According to their various charge they form different moving boundaries in a free electrophoresis as soon as the electric current is started. The electrophoretic analysis is an important method for the characterization of proteins, peptides, nucleic acids, carbohydrates etc., and zone electrophoresis in supporting media such as agar/agarose, starch or polyacrylamide gels is most suitable for separation [6]. For our purpose, i.e. the evaluation of isolated antigens and antibodies (and also conjugates, see later) we employ preferentially agarose and polyacrylamide gels. Moreover, application of special *in situ* characterization reactions for example with histochemical stains [23] enable further information about the chemical nature of the studied material.

Polyacrylamide (PAA) gels are especially suitable supporting media for analytical and preparative separation work due to these characteristics: (1) rapid preparation of variable pore size; (2) reproducible separation; (3) high resolution; (4) easy adaption of chemical, en-

zymatic, lectin and immunological techniques. PAA gels were independently proposed by Raymond and Weintraub [11] and Ornstein [24] and are polymerization products of monomeric acrylamide and the cross-linking monomer N, N'-methylene-bis-acrylamide (BIS) with the following covalent structure [25]:



Chain length and numbers of cross-links determine density, viscosity, elasticity and mechanical stability of the three-dimensional network; free radicals from catalyst-redox systems (from ammonium persulfate, N, N, N', N'-tetramethyl ethylenediamine TEMED) start polymerization [26]. Appropriate variations in gel composition and buffer systems enable electrophoretic separation of molecules not only according to charge but also to their molecular weights (gel gradients, SDS gels) and to their isoelectric points (isoelectric focusing). Hence, special types of PAA gel electrophoreses are known which are performed in flat slabs or in cylindrical glass tubes. The most widely employed methods are the following:

(1) Disc electrophoresis: Ornstein [7] and Davis [28] varied PAA electrophoresis inasmuch as probes in a gel were simultaneously submitted to electric field and pH gradient. Furthermore, discontinuities in buffer composition and pore size of the gel are included. Such discontinuities give, already at the beginning of the run, high concentrated zones which are important for clear-cut separation.

(2) SDS electrophoresis: sodium dodecyl sulfate (SDS) is a potent solubilizing reagent for proteins and in the presence of this anionic detergent, the native charge differences of proteins are masked with the negative charges of SDS and all proteins migrate as anions due to complex formation with SDS. It was observed that an excellent correlation exists between relative migration and logarithm of the molecular weight of proteins [12, 29]. The calculation of unknown molecular weights from proteins is done by comparison of their mobilities with standards of known molecular weight [30].

(3) Pore gradient electrophoresis: the pore size of gels is decreased stepwise or constantly by increasing the degree of crosslinking at high PAA concentrations in the direction of the electrophoretic migration [31 – 33]. Hence, gels can be prepared in which at the beginning of the

electrophorectic run, the mobility of the probes is due to the net charge, and, when the molecules enter the matrix of decreased porosity, their separation is achieved according to molecular weight and conformation.
(4) Isoelectric focusing: the technique of isoelectric focusing enables separation of proteins according to their pI. For this purpose, gels with a stable pH gradient (carrier ampholytes) are prepared which extend from a low pH at the anode to a high pH at the cathode, and proteins are focused in a sharp zone when they reach their pI [34, 35].

The choice of the method depends largely on the material to be studied and the information needed. For routine work, Coomassie Brilliant Blue staining of PAA gels is usual. In certain cases, however, silver staining is to be preferred when very small amounts of proteins should be detected. The use of silver for the detection of proteins in PAA gels becomes widespread and new simplified methods have been developed by which the sensitivity is about 200 times that obtained with Coomassie Brilliant Blue R-250 [36]. Moreover, special techniques can be applied after electrophoresis in order to obtain information about defined physico-chemical characteristics of the separated molecules. Apart from adaption of classical histochemical stainings to PAA gels, binding of antibodies or lectins can provide additional important data about antigenic behaviour and specific carbohydrate composition, respectively. Such binding studies are performed with fluorescent, enzyme or radioactive markers [37].

PAA of isolated alpha-fetoprotein:

1. Alpha$_1$-fetoprotein (AFP) is isolated from mouse and rat amniotic fluids, respectively, by use of solid-phase immunoadsorption (see Chapter 1.2.5).
2. A 7.5 % PAA gel containing 0.1 % SDS is prepared in cylindrical glass tubes and covered with a 4.4 % stacking gel; PAA gels, buffer system and preparation of the probes are according to Glossmann and Lutz [38] and Laemmli [39].
3. After electrophoresis, gels are stained with Coomassie Blue (Fig. 1-1). It can be seen that isolation of mouse and rat AFPs are of high quality. Under the electrophoretic conditions, pure mouse AFP occurs as a single zone, whereas rat AFP shows a characteristic double zone.

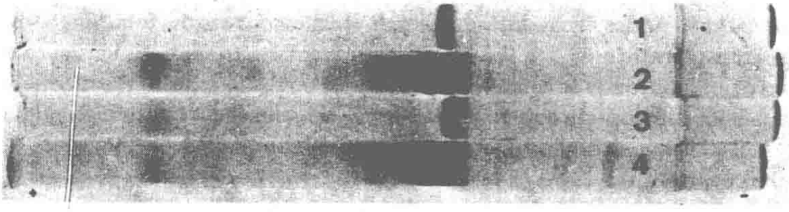


Figure 1-1. PAA electrophoresis; isolated mouse AFP (1), mouse amniotic fluid (2), isolated rat AFP (3), rat amniotic fluid (4).

PAA of isolated intestinal glycoconjugates (acid, Alcian Blue staining):

1. Purification of immunogenic and Alcian Blue reactive acid mucins is done from tissue extracts of a mucus producing carcinoma of human colon. Tumor cells exhibited strong Alcian Blue staining in histology. The principles of antigen purification are described in Chapter 1.2. Briefly, tumor homogenate is submitted sequentially to perchloric acid extraction, ion exchange chromatography, gel filtration and preparative PAA electrophoresis as the final step. From the latter, gel strips of 3 mm were cut and eluted.
2. Eluted fractions are controlled in analytical PAA electrophoresis by use of vertical slab gels [40].

3. Parallel slabs are stained with Coomassie Blue for proteins and with Alcian Blue [41] for acid mucins (Fig. 1-2). It can be seen that effective separation of various acid mucins is obtained with the final preparative PAA electrophoresis. Acid mucins are purified with fractions nos. 4, 5 and 6 which behave in analytical PAA as single-banded and Alcian Blue staining zones. Fractions 4 to 6 showed only very faint staining with Coomassie Blue which is related to the fact that mucins have often a very low proportion of protein. Other preparative PAA fractions (nos. 7 to 10) were either Alcian Blue negative or they were composed of several zones (Coomassie Blue) indicating heterogeneity and ineffective preparative separation.

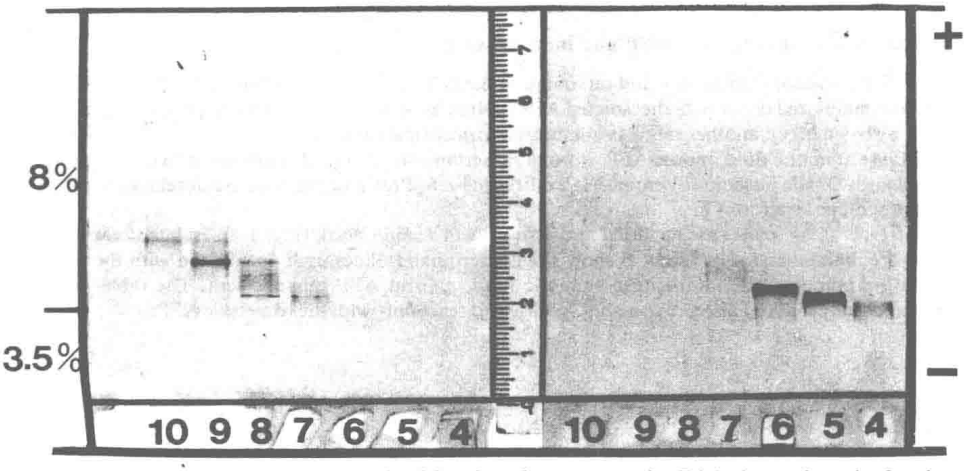


Figure 1-2. Analytical PAA electrophoresis of fractions from preparative PAA electrophoresis; fraction numbers (4 – 10) correspond to eluted gel strips; Coomassie blue (left) and Alcian blue (right) stainings, respectively.

1.1.2 Immunoelectrophorectic analysis and double diffusion in gels

The principle of the various immunological gel diffusion methods is the precipitation reaction which enables the analysis of mixtures of antigens or antibodies (technical preparations in Chapter 8). The immunoelectrophoretic analysis in agar gels was introduced by Grabar and Williams [17] where two different procedures are consecutively performed in a given medium: in the first step, zone electrophoretic separation of the probes; in the second step, diffusion of a precipitating immune serum (from the side and filled into a trough cut in the gel) against an individual , several or the bulk of the antigens (Fig. 1-3).

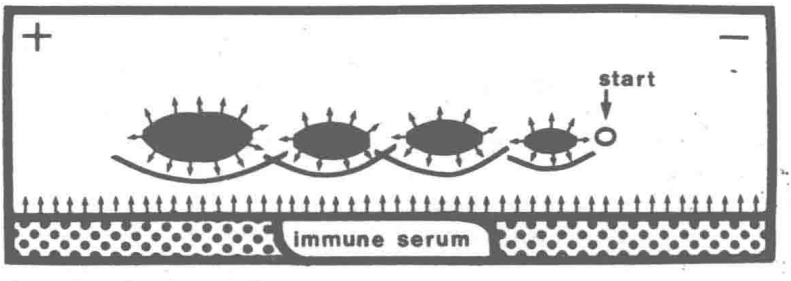


Figure 1-3. Immunoelectrophoresis, schematically.